# Chapter one

## 1.1. Introduction

Antimicrobial resistance is now recognized as one of the most serious global threats to human health in the 21st century (Walsh, 2015). The use, overuse and misuse of antibiotics, as well as their inappropriate disposal, have led to widespread dissemination of antibiotics in various environments, which has resulted in a disproportionate rise in antimicrobial-resistant bacteria that is affecting the treatment of bacterial infections. The widespread emergence of multidrug-resistant (MDR) bacterial pathogens is an important public health challenge worldwide (WHO, 2017). The ABR was developed a post-antibiotic era, means an end to modern medicine and common things as strep throat once again could kill, also some sophisticated interventions like organ transplants, cancer chemotherapy and care of preterm infants would be far more difficult or even dangerous to proceed (WHO, 2017).

*K.pneumoniae* is one of these MDR organisms identified as an urgent threat to human health by the WHO, the US Centers for Disease Control and Prevention and the UK Department of Health. *K. pneumoniae* infections are particularly a problem among neonates, elderly and immunocompromised individuals within the healthcare setting, but this organism is also responsible for a significant number of community-acquired infections including pneumonia and sepsis (Paczosa and Mecsas, 2016). Due to the seriousness of infections caused by MDR *K.pneumoniae* the treatment options are restricted and invariably rely on colistin either singularly or in combination with other antibiotics (Stein and Raoult, 2002; Falagas *et al*., 2011; Biswas *et al*., 2012, Halaby *et al*., 2013).

Colistin, also called polymyxin E belong to the last resort antibiotics known as polymyxins, it is a bactericidal antibiotic produced by *Paenibacillus polymyxa* bacteria, it is effective against most Gram-negative Bacilli (Falagas *et al*., 2009). Colistin is a mixture of polymyxin E1 and E2, two bactericidal lipopeptides, the mode of action of colistin is not fully elucidated but involves binding to a lipopolysaccharides and phospholipids in the outer membrane of Gram-negative organisms, which results in membrane disruption and cell death (Falagas *et al*., 2010). Colistin resistance in *K.pneumoniae* typically occur through mutations in regulatory genes which regulate modification of bacterial targets of polymyxins antibiotics, decreasing the ability of polymyxins to interact (Cannatelli *et al*., 2013; Olaitan *et al*., 2015; Wright *et al*., 2015), other reported resistance mechanisms include shedding of capsular polysaccharides, which trap or bind polymyxins (Padilla *et*

*al*., 2010). The first known plasmid mediated resistance gene to colistin was discovered in *E.coli* strain (strain SHP45) from a big in China on 18 November 2015 during a routine surveillance project on antimicrobial resistance in commensal from food and animals. (Liu *et al*., 2015), it was identified by independent researchers in humans sample from Malaysia, china (Liu *et al*., 2015), United States (McGann *et al*., 2016), Denmark, Netherland, France and Thailand (Olaitan *et al*., 2015). This delicate balance between clinical necessity and prevention of resistance is further compromised by agricultural use of human antibiotics, where some countries have actively used colistin in animal production (Doyle *et al*., 2013).

## 1.2. Rationale

Colistin is an old antibiotic that have recently regained popularity for treatment of severe infections caused by extensively drug-resistant Gram-negative bacteria. As a likely consequence, emergence of colistin resistance is being increasingly reported in the clinical setting, Acquired resistance to polymyxins is generally associated with chromosomal mutations (Falagas *et al*, 2010; Olaitan *et al*., 2015), but a new plasmid-mediated transferable resistance determinant, the *mcr-1* gene, has been described recently. This gene endows resistant against last hope antibiotic-colistin and has ability of horizontal transfer between strains and species; resulting in more aggressive infections (Newton-Foot *et al*., 2017; Wang *et al*., 2018).

*K.pneumoniae* is an important cause of multidrug-resistant infections worldwide. Recent studies highlight the emergence of multidrug-resistant. *K.pneumoniae* strains which show resistance to colistin, a last-line antibiotic, which is a severe problem, especially for critically ill patients, also it's from top three causes of nosocomial and complicated human infections. *K.pneumoniae* have unique genomic plasticity, which position this species as a key amplifier and spreader of AMR genes from environmental sources to human pathogen populations (Kelly and Kathryn, 2018).

In Sudan, there is no information concerning with detection of *mcr-1*gene in *K.pneumoniae*. Therefore, in this study will use PCR for screening the presence of *mcr-1*in *K.pneumoniae* clinical isolates. Discerning more about mechanism of colistin resistance will aid in monitoring the development and spread of resistance.

## 1.3. Objectives

### 1.3.1. General objective

To identify the occurrence of plasmid-mediated colistin resistance gene (*mcr-1*) in *K.pneumoniae* clinical isolates in the Khartoum State, Sudan, using Polymerase Chain Reaction (PCR).

### 1.3.2. Specific objectives

1. To isolate and identify *K.pneumoniae* collected from different clinical specimen (urine and sputum).
2. To determine the sensitivity of *K.pneumoniae* isolates from different clinical specimens towards Colistin.
3. To extract genomic and plasmid DNA from *K.pneumoniae* for Polymerase chain reaction (PCR).
4. To detect plasmid colistin resistance gene *mcr-1* from *K.pneumoniae* clinical isolates.
5. To associate between the presence of *mcr-1* with possible risk factors (gender and site of isolation).

# Chapter Two

# Literature Review

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## 2.1. Historical Background of *K.pneumoniae*

*Klebsiella pneumoniae* belong to a genus *Klebsiella*, it was first described by the German microbiologist Carl Friedlander in 1882 as an encapsulated bacilli after isolating from the fibrous exudate of patients who had died of lobar pneumonia. *K.pneumoniae* is named after the German-Swiss microbiologist and pathologist Edwin Klebs, who was the first to observe bacteria in the airways of patients who died of pneumonia in 1875(CDC, 2014), and it soon became apparent that two bacterial species could cause pneumonia.

## 2.2. General characteristics

Its Gram negative non motile, non-spore forming encapsulated lactose fermenting, facultative anaerobes, rod shaped bacterium. It appears as mucoid lactose fermenter on macConkey agar this phenotype conferred by the polysaccharide capsule attached to bacterial outer membrane. *K.pneumoniae* is most important member of *Enterobacteriaceae* family. According to Ørskov's classification, *K.pneumoniae* may be divided into three subspecies: *K.pneumoniae subsp. pneumoniae, ozaenae and rhinoscleromatis* (Cowan, 1974).

Although found as normal flora of the mouth, skin and intestine (Ryan and Ray, 2004), its naturally occurring in the soil, water, sewage, plant surfaces and animals, and they can colonize medical devices and the health care environment, though capsule type representation differs between clinical/fecal and environmental sources. *K.pneumoniae* is considered as opportunistic pathogen colonizing mucosal surface without causing pathology; however, from mucosa *K.pneumoniae* may disseminate to other tissue causing life threatening infections like pneumonia, blood stream infections, wound infections, urinary tract infections, and meningitis (Paczosa and Mecsas, 2016).

## 2.3. Genomic diversity

*K.pneumoniae* genomes are highly diverse (Lewis, 2013), comprising hundreds of distinct phylogenetic lineages that differ from each other by ~0.5% nucleotide divergence. Individual strains harbor ~2000 'core' (shared) genes, plus a further ~3500 accessory genes that differ between strains and are drawn from a large pool of >30,000 (Lewis, 2013). The~2000 core genes likely facilitate *K.pneumoniae* broad ecological range by providing metabolic and other capabilities enabling survival in a wide range of niches. A substantial

proportion of the total pan-genome (core and accessory genes), (Vernikos *et al*., 2015) is predicted to encode proteins with metabolic functions; 19% associated with carbohydrate metabolism, 18% with other metabolic pathways and 13% with membrane transport (Lewis, 2013). This extensive diversity results in variable metabolic capacity, potentially supplementing individual strains with additional ecological range and providing even more opportunities for genetic exchange. *K.pneumoniae* has a significantly larger genome than the other *Enterobacteriaceae* species, which may help equip *K.pneumoniae* for survival in a broader range of niches. DNA base composition varies widely between taxa, and can be used as a signature of bacterial species (Mann and Chen, 2018). The mean GC content of *K.pneumoniae* core genes is 58%, whereas that of accessory genes ranges from 20% to >70%, suggesting they originate from a taxonomically diverse array of donors (Holt, *et al*., 2015). *K.pneumoniae* receives DNA from a wider diversity of HGT partners; indeed lowest common ancestor analysis of *K. pneumoniae* accessory genes has implicated >20 distinct genera as DNA donors, including numerous other members of the *Enterobacteriaceae* but also diverse groups such as *Acinetobacter*, *Burkholderia*, *Streptomyces*, *Vibrio*, *Xanthomonas* and *Xyella* (Holt *et al*., 2015).

## 2.4. Modes of transmission

*K.pneumoniae* infections transmitted through exposure to the bacteria via respiratory tract, which cause pneumonia, or the blood to cause a blood stream infections. *Klebsiella* infections are most well-known in hospitals spread through person to person contact by contaminated hands of surrounded people in the hospitals, whether it be a healthcare personnel or patients being a significant source. *K.pneumoniae* spread very easily and rapidly, but not through the air. Healthcare settings are most vulnerable to *Klebsiella* infections due to procedures that allow easy access of bacteria into the body. Patients who are on ventilators, catheters, or surgery are highly prone to catching this deadly infection (CDC, 2007).

## 2.5. Pathogenicity and virulence factors

The pathogenicity of *Klebsiella* spp. may be associated with virulence factors, such as capsular polysaccharide antigens; O- and K-antigens, adhesions, siderophores and lipopolysaccharides. LPS also termed endotoxin is a major component decorating the outer membrane of Gram negative bacteria. LPS is widely recognized as the most powerful mediator of septic shock caused by bacteria. Host sensing of LPS via Toll-like receptor 4(TLR4) leads to inflammatory cascade (Roger *et al*., 2009) consist of lipid A, core domain, and O polysaccharide antigen. Variation in O-antigen structure provide various O-

serotypes. In *K.pneumoniae* there are nine main O-serotypes. Three of these, O1, O2, and O3, are responsible for almost 80% of all *Klebsiella* infections (Follador *et al*., 2016). All these component are essentials for the microorganisms to resist complement-mediated killing. Variation in LPS can also play a role in protecting bacteria from antimicrobial peptides, including polymyxin antibiotics (Cheng *et al*., 2015).

Capsular polysaccharides (CPS) is the outer most layer of this pathogen and is involved mainly in resistance to phagocytosis by polymorphonuclear cell by acting as a physical barrier. Some serotypes or capsular types (K-types) of *K.pneumoniae*, e.g. K1, K2, K5, K54 and K57, have been associated with invasive human infectious diseases. K1 was observed among isolates causing Friedlander's pneumonia, and has more recently been associated with pyogenic liver abscesses (Gundestrup *et al*., 2014).

Sidrophore are high affinity, low molecular weight iron-chelating molecules used for acquisition of iron, *K.pneumoniae* secrete multiple types of sidrophores. One of the common cateholates secreted by *K.pneumoniae* is enterobactin which is encoded in core genome (Holden and Bachman, 2015). During recent years, several genes encoding virulence factors in *K.pneumoniae* have been described: the plasmid-borne *rmpA* regulates the mucoid phenotype (Yu *et al*., 2007), *wcaG* is associated with enhanced bacterial escape from phagocytosis (Wu *et al*., 2008). *kfu* is involved in iron acquisition, *fimH* encodes type 1 fimbriae, *mrkD* encodes type 3fimbriae and *cf29A* encodes the non-fimbrial adhesion factor CF29K (Brisse *et al*., 2009).

## 2.6. *K.pneumoniae* infections

*K.pneumoniae* cause serious infections including pneumonia, UTIs, and blood stream infections. An alarming issue and even more common infections originating from *K.pneumoniae* are nosocomial infections (Magill *et al*., 2014). *K.pneumoniae* has been considered opportunistic pathogen, since infection are seen mostly in people with debilitated immune system, these patients population believed to have impaired host defenses, including persons with diabetes, alcoholism, malignancy, liver disease, chronic obstructive pulmonary disease, glucocorticoid therapy and renal failure (Tsai *et al*., 2010).

### 2.6.1. Pneumonia

Historically, *K.pneumoniae* was known as the cause of community-acquired pneumonia or Friedlander's pneumonia, which particularly occurred in immunocompramised persons, such as chronic alcoholics. Friedlander's pneumonia was a severe infection with high mortality if untreated. *K.pneumoniae* is one of a handful bacteria that's is now experiencing

a high rate of antibiotic resistance. Symptoms associated with community acquired pneumonia include sudden onset of, high fever and jelly-like sputum. Shortness of breath and coughing are also common symptoms. These patients have increased tendency to develop lung abscesses, cavitation, empyema, and pleural effusions. If introduced into the blood stream, *K.pneumoniae* has capability of causing meningitis, which effect the CNS. Symptoms include sharp head pain, dizziness, Nausea, and impaired memory.

### 2.6.2. Urinary Tract Infection

UTI alongside bacteremia are leading consequences of *Klebsiella* infections as well, the urinary tract is the most site of infection by *K.pneumoniae.* As with other infections, and UTIs due to *K.pneumoniae* are associated with diabetes mellitus .Catheter-associated UTIs (CAUTIs) are another infection caused by *K.pneumoniae* it's thought that these are facilitated by the ability to form biofilms and adhere to catheters (Schroll *et al*., 2010)..

### 2.6.3. Blood Stream Infection

*K.pneumoniae* are the second leading cause of BSIs caused by Gram -negative bacteria, behind only *E.coli* (Magill *et al*., 2014). *K.pneumoniae* blood stream Infections occur with significant prevalence and high mortality worldwide. The reported mortality rate of k BSIs varies from 15 to 79% (Falcone *et al*., 2016).

### 2.6.4. Pyogenic liver abscess

Pyogenic liver abscess caused by *K.pneumoniae* is an emerging disease worldwide. More lately, On some part of the world, particularly south Asia, highly virulent strains *K.pneumoniae* (often of serotype K1 and ST23) is a cause of community-acquired pyogenic liver abscess with metastatic spread caused by *K.pneumoniae* among previously healthy individuals (Chung *et al*., 2007). Clinical manifestation are characterized by fever and high C-reactive protein, and metastatic infection such as, septic emboli in the lung and endophthalamitis and meningitis are frequently observed (Sifri and Madoff, 2015).

## 2.7. Diagnosis of *K.pneumoniae* infections

*K.pneumoniae* infections usually diagnosed by examining sample of the infected or suspected tissue such as sputum, urine, wound swab, or blood. Depending on the site of infection, which gives clue to the etiology.

### 2.7.1. Gram's Stain

Use of the gram stain techniques shows that bacterial cell wall are red to pink or gram negative, *K.pneumoniae* is large gram negative bacilli non sporing, non-motile and usually capsulated rods (Cheesbrough, 2006).

### 2.7.2. Culture

*K.pneumoniae* are aerobes and facultative anaerobes. Grow readily on ordinary media such as nutrient agar. On blood agar: *K.pneumoniae* produce large grey-white usually mucoid non- hemolytic colonies (Cheesbrough, 2006). MacConkey agar and CLED medium: most *Klebsiella* are lactose-fermenting, producing mucoid pink colored colonies on MacConkey agar and yellow mucoid colonies on CLED medium (Cheesbrough, 2006). After pure culture have been obtained by selecting colonies from aerobically incubated plates, they are identified by biochemical reactions and colony morphology.

### 2.7.3. Biochemical test

Identification of *K.pneumoniae* depends on biochemical characteristics. Which include fermentation of specific sugars (e.g. D-glucose, lactose, sucrose, Larabinose and maltose) and sugar-alcohols (e.g. D-mannitol), utilization of malonate. Production of lysine decarboxylase (but not ornithine decarboxylase), *K.pneumoniae* is indole negative, urease test and citrate utilization test are positive (Cheesbrough, 2006).

Furthermore, *K.pneumoniae* is non-motile and usually produce a prominent acidic polysaccharide based capsule. Biochemical characteristics are still being used for species identification of bacteria isolated from clinical samples.

### 2.7.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) may be performed in several ways. The most common method used for AST in routine laboratories is conventional disk diffusion, which categorize microorganisms as S, I or R. The use of plastic strips, containing an antimicrobial concentration gradient, is a convenient way to generate MIC data on agar plates. Broth dilution is considered the gold standard of MIC determination, but is not commonly used in routine laboratories, Furthermore, automated AST systems (e.g. Vitek2 and Phoenix) are commonly used for AST in routine laboratories, and offer the convenience of combining species identification and MIC determination for relevant agents.

### 2.7.5. Serological test

Are not useful for the detection of infection with *K.pneumoniae* organism.

### 2.7.6. Hematological test

Complete blood count (CBC) usually reveals leukocytosis with left shift, but this is not invariably present.

### 2.7.7. Imaging techniques

#### 2.7.7.1. Chest radiography

The organism usually involves one of the upper lobes; however involvement of other lobes is not uncommon. The affected lobes typically show swollen, cavitation especially in the presence of unilateral necrotizing pneumonia Pleural effusions empyema abscesses formation strongly support the possibility of *K.pneumoniae* infection.

**2.7.7.2 Ultrasounds, X-rays, and computerized tomography** (CT) may also be useful.

## 2.8. Treatment

*K.pneumoniae* can be treated with antibiotics if that infections are not drug-resistant. Unfortunately, *K.pneumoniae* is resistant to a number of antibiotics, deeming treatment options are very limited. Choosing an antibiotics treatment for *K.pneumoniae* depends on the organ system that has been targeted. Antibiotics with high intrinsic activity against *K.pneumoniae* include cephalosporin, Carbapenems, aminoglycosides, and quinolones. Theses treatment are initially used as monotherapy or even as combination. For patients who are severely ill, an initial course, usually between 48-72hours of combination aminoglycosides therapy, is suggested. This should then followed by extended spectrum cephalosporin (Guadalupe *et al.*, 2002).

Carbapenems resistance is an emerging issue and is notably due to *K.pneumoniae* carbapenemases (KPC). With the spread of KPC-producing bacteria, clinicians are dependent on polymyxins for treatment. Polymyxins have been only agents active against KPC- producing bacteria. However, they were used infrequently due to their association with nephrotoxicity and neurotoxicity. This drug often used in combination with other antimicrobials.

Surgery may be needed for patients who experiences empyema, lung abscess, pulmonary gangrene, or respiratory tract obstruction following *K.pneumoniae* infection.

## 2.9. Prevention

Center for disease control and prevention established in 2007 guideline for isolation precautions, preventing transmission, and spreading of infectious agent between healthcare personnel and patients. These precautions may include strict adherence to hand hygiene and wearing gowns and gloves when they enter room where patients with *K.pneumoniae* related illness are housed. To prevent the spread of infections, patients also should clean their hands very often (CDC, 2007), including:

- Before preparing or eating food.
- Before touching their eyes, mouth, or nose.
- Before and after changing wound dressing or bandages.
- After coughing and sneezing.
- After touching hospital surfaces such as bed rails, beside tables, doorknobs, or phones.

## 2.10. Antimicrobial Resistance (AMR)

Antimicrobial resistance is the ability of microbe to resist the effects of medication that once could successfully treat the microbe (WHO, 2015) The term antibiotic resistance (AR) is a subset of AMR but it is apply only to bacteria which becoming resistant to antibiotics. The WHO defines antimicrobial resistance as a microorganism's resistance to an antimicrobial drug that was once able to treat an infection by that microorganisms (WHO, 2015). AMR in bacteria can result both from mutations arising in chromosomal genes and acquisition of mobile genetic elements (MGEs), which harbor AMR genes (Zhang *et al.*, 2011) Screening of AMRs present in bacteria is an important first step to administration of the correct antibiotics to treat bacterial infection.

The antimicrobial resistance crisis facing hospitals globally is driven by the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus; and Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter)*, which are responsible for the majority of infections in hospital patients that are difficult to manage with antimicrobial therapy (Pendleton, *et al*., 2013). ESKAPE pathogens are environmental or commensal bacteria that cause opportunistic infections in hospitalized or immunocompromised patients, but are generally not pathogenic otherwise. Each of these species has intrinsic resistance to one or more antibiotics, and individual strains have accumulated resistance to many additional drugs (Pendleton, *et al.*, 2013). *K.pneumoniae* pathogen is considered the greatest threat (Theuretzbacher, 2017), due to the emergence of strains that are resistant to all or most

available antibiotics (de Man *et al*., 2016). Accumulation of AMR in these organisms is primarily due to horizontal gene transfer (HGT) aided by plasmids and mobile genetic elements (Pendleton, *et al.,* 2013). The catalogue of known mobile AMR genes subject to HGT amongst Gram-negative pathogens numbers in the hundreds (Jia *et al*., 2017). The origins of the AMR genes themselves are environmental bacteria, assumed to be those which have co-evolved with the relevant antimicrobial producing organisms for millennia (Dcosta *et al*., 2011). However, there is typically a lag of several years between the clinical use of a drug and the arrival of relevant mobile AMR genes in human pathogen populations (Lewis, 2013). Hundreds of mobile AMR genes have been found in *K.pneumoniae* (Navon-Venezia, *et al*., 2017).

### 2.10.1. General Causes of Antimicrobials Resistance in Sudan

Of course, the irrational use of antibiotics consider to be the main cause of resistance and this can be in form of unnecessary or over the counter prescription of antibiotics, inadequate use either in dose and/or duration, or overuse of broad-spectrum antibiotics. What makes this dilemma harsher to hinder is the fact that there is no new antibiotics discovered to cope with these new strains of resistant bacteria and no new antibiotics in the pipeline (Greenfacts.org, 2017). Besides the causes that already is mentioned, in Sudan the situation is even more complicated by the general inappropriate use of antibiotics and domination of the wrong concept about antibiotics, for example; using antibiotics for the viral infection, use of antibiotics for curing of most acute health problems and spontaneous antibiotics prescribed when treating pyrexia which is mostly diagnosed as malaria by symptoms with or without confirmation by a laboratory tests, failure in response often can be followed by anti-typhoid treatment based either on false Widal test result interpretation or just persistence of pyrexia, the newest misguided concept is use of antibiotics as prophylactic in many cases example in case of an epidemic disease such as cholera, the other causes are related to local health setting and gaps in protocol and policies such as; lack of regulation for prescriptions and dispensing of the antimalarial agent, easily obtain of antibiotics as self-medication by OTC, lack of surveillance and follow-up, also a shortage of awareness and education programs that targeted simple citizen or not cover regional territories, availability of many brands for the same generic with no clear quality control measurements. Also the use of sub-therapeutic dose for animal growth promotion and misuse of antibiotics in the routine animal treatment (Ahmed *et al.*, 2016).

### 2.10.2. Genetic basis of antimicrobial drug resistance

Bacteria use two major genetics strategies to adapt to the antibiotic, Mutations in genes often associated with mechanisms of action of the compound and acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT).

#### 2.10.2.1. Mutational Resistance

Subset of bacterial cells derived from a susceptible population develop mutations in genes that affect the activity of the drug, resulting in preserved cell survival in the presence of the antimicrobial molecule. Once a resistant mutant emerges, the antibiotic eliminates the susceptible population and the resistant bacteria predominate. In many instances, mutational changes leading to resistance are costly to cell homeostasis and are only maintained if needed in the presence of the antibiotic. In general, mutations resulting in antimicrobial resistance alter the antibiotic action via one of the following mechanisms:

- Production of enzymes that makes modifications of the antimicrobial target (decreasing the affinity for the drug).
- In the drug uptake, changing the permeability a decrease one of the drugs that associated with a change in permeability of the drug.
- Chemical destroy the active drug: Gram-negative rods resist aminoglycosides by production adenylating, phosphorylating or acetylating enzyme that destroys the drug.
- Activation of efflux mechanisms to extrude the drug from the cell surface (Li and Nikaido, 2009)

#### 2.10.2.2. Horizontal Gene Transfer (HGT)

The transfer of foreign genes between organisms is referred to as horizontal gene transfer (Heuer and Smalla, 2007). Acquisition of foreign DNA material through HGT is one of the most important drivers of bacterial evolution and it is frequently responsible for the development of antimicrobial resistance. Most antimicrobial agents used in clinical practice are products naturally found in the environment (mostly soil). Bacteria sharing the environment with these molecules harbor intrinsic genetic determinants of resistance and there is robust evidence suggesting that such “environmental resistome” is a prolific source for the acquisition of antibiotic resistance genes in clinically relevant bacteria. There are three mechanisms of HGT: Transformation is the active uptake of free DNA from the environment by competent bacterial cells, Transduction is an infection of bacteria by

bacteriophages or bacterial viruses, who inject foreign DNA into a bacterial cell, Conjugation is the mechanism where a donor and a recipient cell physically connect to each other and share DNA carried by conjugative elements, such as plasmids and transposons (Heuer and Smalla, 2007). Furthermore, this genetic exchange has been implicated in the dissemination of resistance to many used antibiotics.

## 2.10.3. Mechanisms of Antimicrobial Resistance

Not surprisingly, bacteria have evolved sophisticated mechanisms of drug resistance to avoid killing by antimicrobial molecules, a process that has likely occurred over millions of years of evolution. Of note, resistance to one antimicrobial class can usually be achieved through multiple biochemical pathways, and one bacterial cell may be capable of using a cadre of mechanisms of resistance to survive the effect of an antibiotic.

**2.10.3.1. Modifications of the Antibiotic Molecule**: The production of enzymes capable of introducing chemical changes to the antimicrobial molecule is a well-known mechanism of acquired antibiotic resistance in both Gram-negative and Gram-positive bacteria. Interestingly, most of the antibiotics affected by these enzymatic modifications exert their mechanism of action by inhibiting protein synthesis at the ribosome level (Wilson, 2014). Many types of modifying enzymes have been described, and the most frequent biochemical reactions they catalyze include acetylation in aminoglycosides, chloramphenicol and streptogramins, phosphorylation in aminoglycosides, chloramphenicol, and adenylation in aminoglycosides and lincosamides.

**2.10.3.2. Destruction of the antibiotic molecule:** The main mechanism of β-lactam resistance relies on the destruction of these compounds by the action of β- lactamases. These enzymes destroy the amide bond of the β-lactam ring, rendering the antimicrobial ineffective.

**2.10.3.3. Decreased Antibiotic Penetration and Efflux:** this mechanism Acts as the first-line of defense against the penetration of multiple toxic compounds, including several antimicrobial agents. Hydrophilic molecules such as β-lactams, tetracyclines and some fluoroquinolones are particularly affected by changes in permeability of the outer membrane since they often use water-filled diffusion channels known as porins to cross this barrier (Pages, *et al.*, 2008). The prime example of the efficiency of this natural barrier is the fact that vancomycin, a glycopeptide antibiotic, is not active against gram-negative organisms due to the lack of penetration through the outer membrane.

**2.10.3.4. Efflux Pumps**: The production of complex bacterial machineries capable to extrude a toxic compound out of the cell can also result in antimicrobial resistance. The description of an efflux system able to pump tetracycline out of the cytoplasm of *E. coli* dates from the early 1980s and was among the first to be described (McMurry, *et al.*, 1980). Since then, many classes of efflux pumps have been characterized.

**2.10.3.5. Modification of the Target Sites:** Bacteria have evolved different tactics to avoid the action of the antibiotic, by preventing the antibiotic to reach its binding site that result in decreased affinity for the antibiotic molecule an example of target protection is the quinolone resistance protein *Qnr*, which is a plasmid-mediated fluoroquinolone resistance. Initially described in a clinical isolate of K. pneumoniae in the mid-1990s (Martinez-Martinez et *al.*, 1998), its acts as a DNA homologue that competes for the DNA binding site of the DNA gyrase and topoisomerase IV. It is thought that this reduction in the DNA gyrase-DNA interaction decreases the opportunities of the quinolone molecule to form and stabilize the gyrase-cleaved DNA quinolone complex that is lethal for the cell (Rodríguez-Martinez *et al*., 2011)

**2.10.3.6. Mutations in the genes encoding the target site:** FQs kill bacteria by altering DNA replication through the inhibition of two crucial enzymes, DNA gyrase and topoisomerase IV. Development of chromosomal mutations in the genes encoding subunits (*gyrA-gyrB and parC-parE* for DNA gyrase and topoisomerase IV, respectively) is the most frequent mechanism of acquired resistance to these compounds.

**2.10.3.7. Enzymatic alterations of the binding site**: Addition of methyl groups. e.g. the methylation of the ribosome catalyzed by an enzyme encoded by the *erm* genes (erythromycin ribosomal methylation), which results in macrolide resistance.

## 2.11. Emergence of antibiotic resistance in *K.pneumoniae*

There are multiple factors believed to contribute in the spread of antibiotic resistance, including inappropriate antibiotic use in healthcare, and lack of new antimicrobial therapeutics (CDC, 2014). Several mechanisms of antibiotics resistance are found in *K.pneumoniae*, with resistance to b-lactam having the greatest impact on effective treatment. Colonization with antibiotic-resistant *K.pneumoniae* has been associated with subsequent infection with this bacterium in hospitalized patients.

### 2.11.1. B -lactamase-producing *K.pneumoniae*

Resistance to β -lactam antibiotics observed before penicillin was widely used to treat infection. In 1929 Alexander Fleming was first to note that *E.coli* and other bacteria are

not inhibited by penicillin, latterly this resistance particularly related to enzymes produced by these bacteria (Abraham and chain, 1940). Mechanism of resistance to this family of antibiotics through hydrolysis of amide bond of the β-lactam ring by -lactamases.
Resistance to β-lactam antibiotics in *K.pneumoniae* obtained intrinsically since the enzyme encoded in the core genome. For example, SHV is consistently found in chromosome, and cross-bonding ampicillin resistance is hallmark of this species (Bialeck-Davenet *et al*., 2014). In 1960s the first plasmid–mediated β-lactamase, *TEM-1*, was discovered in *E.coli* (Datta and Kontomichalou, 1965), it was isolated in Athens from a patient called Temoneira, and thus named *TEM-1* after this patient. *TEM-1*-producing *K.pneumoniae* became endemic in many hospitals (Medeiros, 1997).

*K.pneumoniae* also harbor plasmid-mediated β-lactamase, such as *AmpC* enzyme which is cephalosporinase that generally confer resistance to most of penicillin and cephalosporin antibiotics (Jacoby, 2009). From then on, the development of newer generations of β-lactams has systematically been followed by the rapid appearance of enzymes capable of destroying any novel compound that reach the market, in a process that is a prime example of antibiotic driven adaptive bacterial evolution.

### 2.11.2. Extended Spectrum β-lactamases producing *K.pneumoniae* (ESBLs)

Extended spectrum β-lactamases ESBLs are enzyme has the ability to hydrolyze penicillin, third generation Cephalosporins (the hallmark characteristic) and monobactams, but harbor modest activity against Cephamycins and Carbapenems. ESBLs are plasmid based resistance mechanisms, CTX-M is a plasmid-encoded ESβL. CTX-M enzymes have become the most prevalent ESBL worldwide and are responsible for a large proportion of cephalosporin resistance in *E.coli* and *K.pneumoniae*. In 1983 was the first ESBL-producing *K.pneumoniae* was identified by knothe et al in Europe.

### 2.11.3. Carbapenems-resistant *K.pneumonia*e (KPC)

Possibly due to the selective pressure of treating ESBLs infection with Carbapenems, Carbapenems-resistant has emerged. A diverse group of β-lactamases with the ability to significantly hydrolyze Carbapenems at least Imipenem and/or Meropenem (Nordmann and Poirel**,** 2002**)**, the most potent β-lactams available in clinical practice.

*K.pneumoniae* the most common Carbapenems-resistant Enterobacteriaceae (CRE) KPC was first reported in 1996 from a *K.pneumoniae* recovered from a patient in North Carolina, USA (Yigit *et al.,* 2016). Although these enzymes are predominantly found in *Klebsiella*

spp. (therefore its name, *Klebsiella pneumonia* carbapenemases). More recently in 2008, a new carbapenemase was identified in a *K.pneumoniae* isolate recovered from a Swedish patient who had been previously admitted to a hospital in New Delhi, India. The enzyme was designated *NDM-1*, in reference to its origin (New Delhi Metallo β-lactamase) (Kumarasamy *et al.*, 2010). *K.pneumoniae* is responsible for 80% of CRE infections (CDC, 2014). One possible explanation of is that CPE is still common among *K.pneumoniae*, which is the bacteria most commonly acquired in community.

Oxacillinase-type β-lactamase (*OXA-48*) enzyme was for the first identified in a Carbapenem-resistant *K.pneumoniae* in turkey in 2001, which was found to be multidrug resistant, including resistance to the Carbapenems (Poirel et *al.*, 2004).

### 2.11.4. Colistin resistance in *K.pneumoniae*

Colistin resistance in *K.pneumoniae* typically occur through regulatory genes such as *mgrB* that regulate the modification of bacterial lipid A, the target of polymyxins antibiotics reducing its ability to interact with bacterial membrane (Cannatelli *et al*., 2013; Olaitan *et al*., 2015*;* Poirel *et al*., 2015*;* Wright *et al*.,2015). Recently, a plasmid-borne transferase *mcr-1* has been detected in KPC-producer isolated from an infected wound. The clinical consequence of dissemination of KPC harboring *mcr-1* could be devastating due to the risk of total lack of effective antibiotic treatment against such infections.

## 2.12. Colistin

Colistin, a polymyxin, was used from the 1960s to the early1980s. Because toxicity considerations (mainly nephrotoxicity neuromuscular blockade, and neurotoxicity), its systemic use has been all but abandoned (Catchpole, *et al*., 1997).

Colistin belongs to a group of antimicrobial agents known as polymyxins discovered in 1947 (Benedict and Langlykke, 1947; Ainsworth *et al*., 1947). They are a class of cationic antimicrobial peptides, which were originally isolated from the spore-forming soil organism *Paenibacillus polymyxa.* Molecules in this group are polymyxins A, B, C, D and E, of which only polymyxin E (Colistin) and polymyxin B were used clinically in humans. Colistin is a mixture of polymyxin E1 and E2, two bactericidal pentacationic lipopeptides. The mode of action of Colistin is not fully elucidated but involves binding to lipopolysaccharides and phospholipids in the outer membrane of gram-negative organisms, which results in membrane disruption and cell death (Hancock, 1997), and is not active against gram-positive bacteria, which lack an outer membrane.

Colistin used in both human and veterinary medicine. In humans, Colistin is generally used to treat infections with multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria (Magiorakos *et al.,* 2012). It is usually administered by injection or inhalation in case of cystic fibrosis, as the sodium salt of Colistin methanesulfonate, which is an inactive pro-drug. It is considered less toxic than Colistin sulfate, which is used orally (with very limited absorption) or topically (Falagas and Kasiakou, 2005). Resistance to Colistin can be acquired in some bacteria such as in *P.aeruginosa* and *K.pneumoniae*, whereas bacteria such as *Proteus species* and *Serratia species* are naturally resistant to these drugs. Reports of Polymyxins resistance in clinical isolates have increased recently, for both acquired and intrinsically resistant bacteria. This becomes a worse nightmare, due to the low number of currently available effective antibiotics (Olaitan *et al*., 2015).

### 2.12.1. Mechanisms of polymyxin resistance

Known resistance mechanisms include intrinsic, mutational and adaptive, but recently, horizontally acquired resistance has also been described (Hood *et al*., 2013; Liu *et al*., 2016).The major polymyxins resistance mechanisms include: Alteration of the lipopolysaccharide (LPS) moiety, resulting in a reduction of the net LPS negative charge,Mutations in genes, Increased drug efflux., Reduced porin pathway, Formation of capsules and enzymatic inactivation of Colistin. However, other reported resistance mechanisms include shedding of capsular polysaccharides, which trap or bind polymyxins, as found in some isolates of *K.pneumoniae* (Olaitan *et al.*, 2015).

## 2.13. Plasmid

Plasmids are small, circular pieces of double- stranded DNA that is distinct from a cells chromosomal DNA. Plasmids naturally occur in bacterial cell, and they also occur in some eukaryotes. Plasmids have a wide range of lengths from a few thousand base pairs to more than hundreds kilobases (Kb).when a bacterium divides, all of the plasmids contained within the cell are copied such that each daughter cell receives a copy of each plasmid. The genes carried in plasmids provide bacteria with genetic advantages, such as antibiotics resistance. Such drug- resistance plasmids have become a major problem in a treatment of common bacterial pathogens. As antibiotics use became wide spread plasmids containing several drug- resistance genes evolved, making their host cells resistant to a variety of different antibiotics simultaneously. Many of these plasmids also contain “transfer genes” encoding proteins that can form macromolecule tube, or pilus through which a copy of the plasmid can be transferred to other host cells of the same or related bacterial species which known as conjugation (Smillie *et al*., 2010). Such transfer can result in the rapid spread of

drug- resistance plasmids, expanding the number of antibiotics- resistant bacteria in an environment such as a hospital. The vast majority of AMR genes in *K.pneumoniae* are plasmid-borne (Navon-Venezia, *et al.*, 2017; Wyres and Holt, 2016), hence the ability to amplify and spread AMR genes across ecological niches is likely linked to plasmid-permissive traits. Highly diverse environmental microbial communities, especially soils, are considered hotspots for gene transfer (Popa *et al*., 2011). The gene of *mcr-1* was first recovered from plasmid pHNSHP45 which are fully sequenced and identified. Other types of plasmids also have been identified (Liu *et al*., 2016). Coping with the spread of drug-resistant plasmids is an important challenge for modern medicine.

## 2.14. The discovery and dissemination of *mcr-1*

Previously, chromosomally-encoded PEtN transferases have been observed to be under the control of the *PhoP-PhoQ* and *PmrA-PmrB* pathway, until the recent discovery by Liu *et al.* that documented the emergence of a plasmid-mediated polymyxin resistance mechanism catalyzed by a PEtN transferase enzyme called *mcr-1*(Liu *et al*., 2015).

A plasmid-mediated gene that confers resistance to Colistin, *mcr-1*, was first reported In November 2015 in *E. coli* isolates from food animals and their meat collected in China during 2011– 2014 and in *E.coli* and *K.pneumoniae* isolates collected from human patients in China in 2014 (Liu *et al.,* 2015), *mcr-1* an acronym for mobilized Colistin resistance gene, it's protect bacteria from a polypeptide antibiotic Colistin. Its gene code for an enzyme called phosphotidylethanolamine transferase which transfer the phosphotidylethanolamine residue to the lipid A of the gram negative bacterial cell membrane. The lipid A of the bacterial outer membrane is the target binding site for Colistin, which upon binding to it, disrupts its by displacing magnesium and calcium, thus causing cell death.

*mcr-1* has been found to be located on a pHNSHP45plasmid, its presence in a plasmid; has the potential to quickly spread to other bacteria due to the ability of Gram-negative organisms to readily take up foreign DNA and a plasmid-mediated resistance mechanism to one of the most potent classes of antibiotics has the potential to create an enormous impact on the future use of polymyxin as an antibiotic and raises the possibility that bacteria already resistant to major antibiotics could become resistant to Colistin as well (Liu *et al*., 2015). Colistin had been used extensively in food-producing animals globally for many years, it was not available for human clinical use in China until 2017, suggesting that the selection pressure for the spread of *mcr-1* was driven by

veterinary use of Colistin (Skov and Monnet, 2016). The spread of *mcr-1* via horizontal gene transfer to other MDR bacteria has been observed (Falgenhauer, *et al.*, 2016).

**2.15. Previous studies**

Shortly after its first description, it was observed that the *mcr-1* gene had spread globally in *K.pneumoniae, E.coli*, *Enterobacter cloacae* and *Salmonella spp* isolates of animal, environmental and human origin (Liu *et al.*, 2015; Doumith *et al*., 2016; Al-Tawfiq *et al.*, 2017), posing a threat to the longevity of polymyxins in the clinic. Additionally, one report suggests that the gene has been identified in at least 16 countries (Ye *et al.*, 2016). The global spread of *mcr-1* was probably also facilitated by human travel, as suggested by the finding of the gene in enteric bacteria from travelers returning to Europe after visiting countries with a high prevalence of *mcr-1* in South America, Asia and Africa (Skov and Monnet, 2016; Arcilla, 2016). In Italy, the proportion of Colistin resistance among KPC-producing *K.pneumoniae* was (57%) in 2013 (Gianni *et al*., 2015).

After identification of *mcr-1,* wide scientific attention led to recognition of multiple *mcr-1* variants (Di Pilato, 2016; Partridge *et al.,* 2018) and seven additional *mcr* gene families (Xavier *et al.*, 2016). The latter include *mcr-2* in *E. coli* and *Salmonella* spp. from pigs, cattle and chickens in Belgium (Garcia-Graells *et al.,* 2018; Yin *et al.,* 2017), *mcr-3* in human and animal *E. coli*, *Proteus* spp, and *Aeromonas* spp. in countries in Asia, Europe and South America (Carretto *et al.*, 2016), *mcr-4* in *E. coli* and *Salmonella enterica* serovar *Typhimurium* from pigs and humans in Belgium, Italy and Spain (Carretto *et al.,* 2016; Borowiak *et al.,* 2017), *mcr-5* in poultry and poultry meat isolates of *S.enterica* serovar Paratyphi B d-tartrate fermenting and in porcine *E. coli* in Germany (Hammerl *et al.,* 2018; Yang *et al.,* 2018), *mcr-6* in *Moraxella* sp. isolates from pigs in the United Kingdom (Partridge *et al.,* 2018), *mcr-7* in *K.pneumoniae* from chickens in China (Wang *et al.*, 2018) and *mcr-8* in *K.pneumoniae* from humans and pigs in China (Litrup *et al.,* 2017). The co-occurrence of more than one of these genes has been reported in *E. coli* and *Salmonella* spp. Isolates of human and animal origin in Europe and China, such as the simultaneous presence of *mcr-1* and *mcr-3* and of *mcr-1* and *mcr-4* (Hernández *et al.*, 2017; Karvanen *et al.,* 2017). Bacteria isolated from food-producing animals to date appear to carry the *mcr* genes more frequently than bacteria isolated from humans, which is probably a consequence of the selective pressure exerted by the wide use of Colistin in veterinary practice (Olaitan *et al.,* 2015). Importantly, *mcr*-mediated Colistin resistance can be transferred among bacterial strains, species and genera. To limit further dissemination of such genes, accurate identification of Colistin resistant, *mcr*-encoding isolates is of critical importance.

# Chapter Three

# Materials and methods

# Chapter Three

# Materials and Methods

## 3.1. Study design

It's a descriptive cross sectional and hospital-based study to detect the presence of *mcr-1*gene from clinical isolates of *K.pneumoniae*.

## 3.2. Study area and duration

The study was carried out in Khartoum State. The PCR was performed at research laboratory in Sudan University of Sciences and Technology, during the period from June to November, 2019.

## 3.3. Study population

*K.pneumoniae* clinical isolates were obtained from different hospitals: (Military hospital, Police University Hospital, Royal Care Hospital).

## 3.4. Sample size

A total of 50 isolated *K.pneumoniae* from clinical specimens, urine and sputum.

## 3.5. Sampling techniques

The study was based on convenience non probability sampling technique.

## 3.6. Data collection

A questionnaire (personal information includes name, age, and gender and isolation site) was designed and used in the study.

## 3.7. Ethical consideration

This study was approved by the Ethical and Scientific Committee from College of Medical Laboratory Sciences, Sudan University for Sciences and Technology.

## 3.8. Laboratory methods

### 3.8.1. Growth examination materials

Standard amount of preserved *K.pneumoniae* isolates were inoculated separately into this suitable media.

### 3.8.2. Cysteine Lactose Electrolyte Deficient Agar media (CLED)

CLED (HI-MEDIA, India) used for isolation of Gram-negative bacteria and to differentiate lactose fermenting organisms from non-lactose fermenting organisms (Mackie *et al*., 1996).

### 3.8.3. Muller Hinton agar

This media (HI-MEDIA, India) was used for antimicrobial susceptibility testing (Mackie *et al*., 1996).

### 3.8.4. Nutrient agar

This media (HI-MEDIA, India) was used for purification and short preservation of organisms (Mackie *et al*., 1996).

### 3.8.5. Biochemical test

The following tests have been done according to standard laboratory procedures (Cheesbrough, 2006). Using sterile straight wire loop, the colonies were touched and inoculated on (Kliger Iron Agar, tryptophan peptone water, Simmons's citrate agar, Christensen's urea agar) (HIMEDIA, India), and then incubated at 37 ºC overnight incubation, then interpreted according to their reactions.

**Indole test**

A sterile loop was used to inoculate the tested organism into (2 ml) peptone water; the tube was incubated at (37°C) for (24 hrs.). In the next day, add drop of Kovac's reagent (4 (p) – dimethylamino benzaldehyde) was added, it was shacked gently and examined for a red color ring within (10 mints). *K.pneumoniae* is negative for Indole test (Cheesbrough, 2006).

**Kligler Iron Agar (KIA)**

The tested organism was inoculated into KIA medium, using a straight wire, the agar butt was stabbed, the opening was closed and then the top slope was streaked (as zigzag). The medium was incubated at (37 °C) for (24 hrs.). Glucose fermentation, lactose fermentation, $H_2S$ production and gas production were reported. *K.pneumoniae* gave yellow butt and slope produce no gas or $H_2S$ (Cheesbrough, 2006).

**Urease test**

The test organism was cultured in a medium which contains urea, and the indicator phenol red, by using a sterile straight wire and under a septic condition, the media was inoculated

and incubated overnight at 37°C, *K.pneumoniae* are Urease positive produce pink color (Cheesbrough, 2006).

**Citrate utilization test**

The tested organisms were suspended in a normal saline and cultured in a Simmons's citrate agar. By using a sterile straight wire, firstly the slope was streaked and then butt was stabbed and incubated overnight at 37°C. *K.pneumoniae* gave positive result (bright blue) (Cheesbrough, 2006).

### 3.8.6 *In- Vitro* antibiotic sensitivity testing

Susceptibility pattern was done by disk diffusion method. All the isolated organisms were put into appropriate media for antibiotic susceptibility test. Disc diffusion tests performed and interpreted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2012). All tests were performed on Muller Hinton agar plates. The surface was lightly and uniformly inoculated by sterile cotton swab stick. Prior to inoculation, the swab stick was dipped into a bacterial suspension having visually equivalent turbidity to (0.5) McFarland standards. The swab stick was then taken out and squeezed on the wall of the test tube to discard extra suspension, after inoculation the following discs (HI-MEDIA, India) were placed; Amoxicillin (25µg), Ciprofloxacin (5µg), Amikacin (10µg), Ceftazidime (30µg), Imipenem (10µg) and Colistin (10µg). Inoculated plates were incubated at (37ºC) for (24hrs). On the next day, Inhibition zones were measured in millimeter (mm) by using a ruler over the surface of the plate with the lid open. They were held a few inches above a black, nonreflecting background and illuminated with reflected light. Results were recorded and graded as resistant (R) and sensitive (S), according to the reference zone of inhibition of particular antibiotic (CLSI, 2012).

## 3.9. Identification of mobile Colistin resistance gene (*mcr-1*) by polymerase chain reaction (PCR)

### 3.9.1. Extraction of DNA for the PCR

DNA was extracted by using boiling method; two colonies of overnight growth bacteria were used. The colonies were dissolved in 1ml of distilled water in 1.5 eppendorff tube and boiled for 10 minutes in a water bath, and then were centrifuged for 5minutes at 1000 rpm, and the supernatant were used as template DNA for the PCR.

## 3.9.2. PCR primers for identification of *mcr-1* gene

**Table 3-1:** Primers Sequences used for the Amplification of *mcr-1* Gene (Liu *et al*., 2015)

| **Target gene** | **Primer name** | **Sequence(5'- 3')** | **Amplicon size(bp)** |
|---|---|---|---|
| ***mcr-1*** | ***mcr-1F*** | **5'-CGGTCAGTCCGTTTGTTC-3'** | **309bp** |
| | ***mcr-1R*** | **5'-CTTGGTCGGTCTGTAGGG-3'** | |

## 3.9.3. Primers Preparation

### 3.9.3.1. Stock primer

Centrifugation of primer vial was done firstly then (250µl) of sterile DW was added to each vial.

### 3.9.3.2. Working Primer

From each stock primer (10µl) was dissolved in (90µl) of sterile DW and stored at (-20 ºC).

### 3.10. Master Mix

Master Mix kits (INtRON Maxime PCR PreMix, Korea) containing all reagents for PCR except water, template and primers were used. Storage of the master mix was carried out at (-20˚C).

### 3.10.1. Preparation of Reaction Mixture:

**Table 3-2:** Preparation of Reaction Mixture for *mcr-1* Gene Amplification.

| **Reagents** | **Volumes** |
|---|---|
| Master mix premix(i-Taq) | 5 ml |
| Distilled water | 5 ml |
| Forward primer | 0.5µl |
| Reverse primer | 0.5µl |
| DNA template | 2µl |

## 3.11. Amplification Conditions of PCR

The amplification was done by using (0.2) PCR Eppendorf tubes that subjected to thermocycler PCR (SensoQuest, Germany). The amplification conditions listed in (Table 3-3).

**Table 3-3:** PCR Amplification conditions

| PCR cycle | Temperature C° | Time | No of cycle |
|---|---|---|---|
| **Initial denaturation** | 94°C | **5min** | **1** |
| **Denaturation** | 94 °C | **1min** | **35** |
| **Annealing** | 52 °C | **1min** | |
| **Extension** | 72 °C | **1min** | |
| **Final Extension** | 72 °C | **10 min** | **1** |

### 3.12. Gel Electrophoresis

#### 3.12.1. Preparation of Agarose Gel

Amount of (1.5 gm) of agarose powder (BIOLINE, UK) was weighted, (100 ml) 1X TBE buffer (Sigma, Japan) was added, the mixture was heated by microwave until clear solution is produced, allowed to cool, then (2µl) of Ethidium bromides were added, mixed well and poured onto suitable gel tray that was equipped with suitable combs to form well that used for loading the PCR products. Any bubbles were removed and the gel was allowed to solidify at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed.

#### 3.12.2. Electrophoresis of the Samples

The gel casting tray was flooded by 1X TBE buffer near the gel cover surface, then (4µl) of PCR products of each sample was loaded into each well, then to the first well of casting tray (2 µl) of (100bp) DNA ladder (marker)(INtRON) was injected for each run. The gel electrophoresis apparatus was connected to the power supply power supply(CONSORT E865, Belgium).Then the electrophoresis was run at (100) volts for (15 minutes).

#### 3.12.3. Visualization of the Samples

The gel was removed by gel holder and visualized under the ultraviolet Trans- illuminator (UVitec UK), to detect the specific amplified products by comparing with 100 base pairs standard ladders.

### 3.13. Data Analysis

All outcome data were analyzed by using Statistical Package for Social Sciences (SPSS; Version20). Using Chi square test to estimate *p* value of significance $\leq 0.05$. The outcome data arranged in tables and then entered into SPSS according to program guidelines.

# Chapter four

# Results

# Chapter four

## 4. Results

A total of (50) *K.pneumoniae* isolates were collected from Royal care hospital, Military hospital, Police University Hospital at Khartoum State from June 2019 to September 2019. All Isolates were re-identified as *K.pneumoniae* by molecular technique. The distributions of enrolled patients were 35 (70%) males and 15 (30%) females as shown in table (4-1)

**Table (4-1):** distribution of enrolled patients in the study

| Gender | Number | Percentage |
|---|---|---|
| Male | 35 | 70% |
| Female | 15 | 30% |
| Total | 50 | 100% |

### 4.1. Distribution of the Isolates according to the site of infection

The specimens were collected from different sites; urine 32(64%), sputum 18 (46%) as shown in (table 4-2). *mcr-1* was only found in urine specimen and it was 2(4%) as shown in figure (4-2).

Statically there was no association between the presence of *mcr-1* gene and the site that from which sample was isolated (p-value = 0.3).|

**Table (4-2):** Show the distribution of the Isolates according to the site of infection.

| Type of specimen | Number | Percentage% |
|---|---|---|
| **Urine** | 32 | 64% |
| **sputum** | 18 | 46 % |

## 4.2. Antimicrobial Susceptibility Test

Table (4-3) revealed Antimicrobial susceptibility and resistance pattern to Colistin (10µg), Imipenem (10µg), Ciprofloxacin (5µg), Amikacin (10µg), µg), Amoxicillin (25µg), and Ceftazidime (30 µg). Notably that there is 6 (12%) out of 50 isolates were resist to Imipenem and 8 (16%) isolates were resist to Colistin as shown in (table 4-3).

**Table 4-3:** Antimicrobial susceptibility pattern of *K.pneumoniae* (N=50).

| **Antimicrobial Agent** | **Resistant Isolates** | | **Susceptible Isolates** | | **Total** |
|---|---|---|---|---|---|
| | Number | % | Number | % | |
| **Imipenem** | 6 | 12% | 44 | 88% | 50 |
| **Colistin** | 8 | 16% | 42 | 84% | 50 |
| **Amikacin** | 34 | 68% | 16 | 32% | 50 |
| **Ciprofloxacin** | 43 | 86% | 7 | 14% | 50 |
| **Ceftazidime** | 50 | 100% | 0 | 0% | 50 |
| **Amoxicillin** | 50 | 100 | 0 | 00% | 50 |

## 4.3. Amplification of *mcr-1* gene

For PCR, *mcr-1* backward and forward primers mentioned in Table (3-1) were used to amplify *mcr-1* gene region, each amplicon was run on (1.5%) agarose gel. *K.pneumoniae* isolates that were successfully amplified gave a band of (309 bp) when the gel was visualized under UV light, this was considered as positive result for *mcr-1*gene presence.

## 4.4. Detection of *mcr-1* gene among *K.pneumoniae* isolates

PCR amplification showed that only 2 (4%) out of (50) *K.pneumoniae* clinical isolates were found to carry *mcr-1* gene as shown on (figure 4-1).

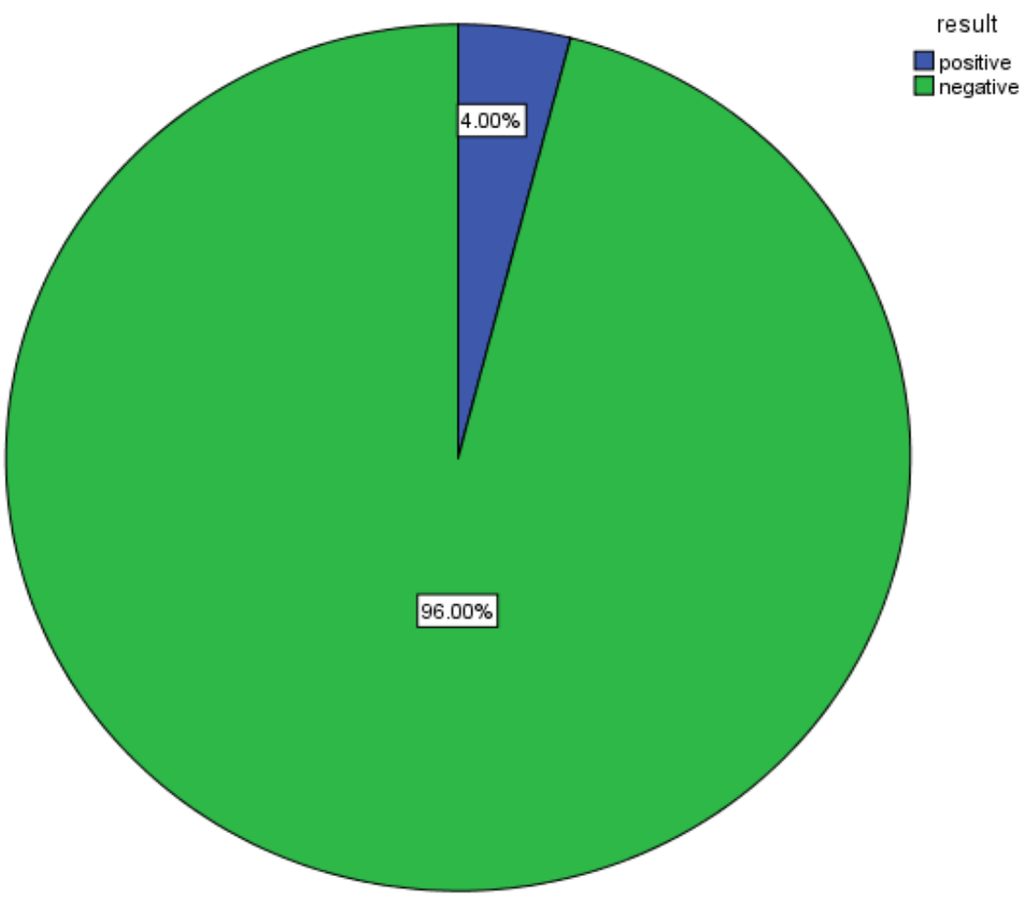



**Figure 4-1: Distribution of *mcr-1*gene Positive *K.pneumoniae isolates*.**

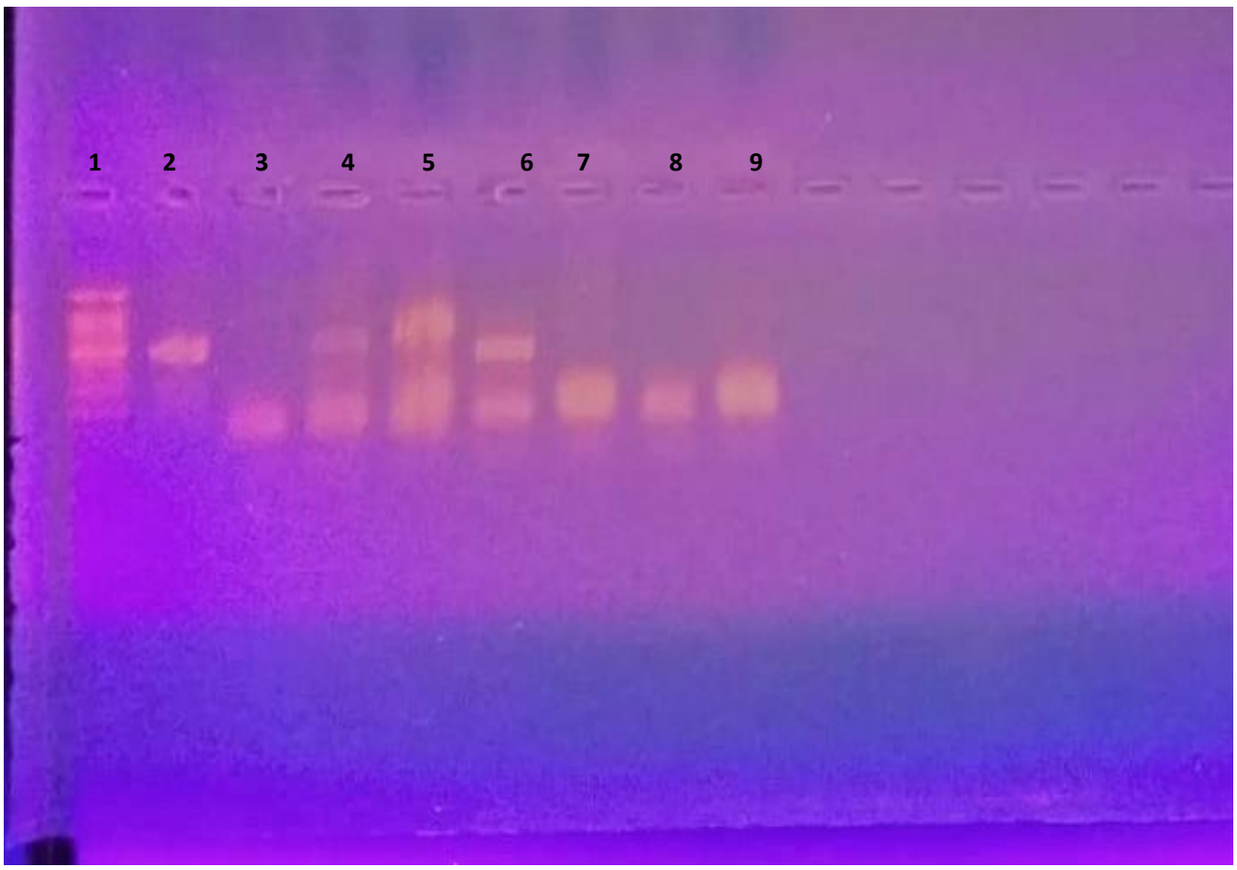



**Figure 4-2: Agarose Gel Electrophoresis of PCR product *mcr-1* gene of *K.pneumoniae***: lane **1**:100bp ladder, lane **2, 3, 4, 5**: negative sample, lane **6**: band of *mcr-1* gene (309bp), lane **7, 8, 9** negative sample**.**

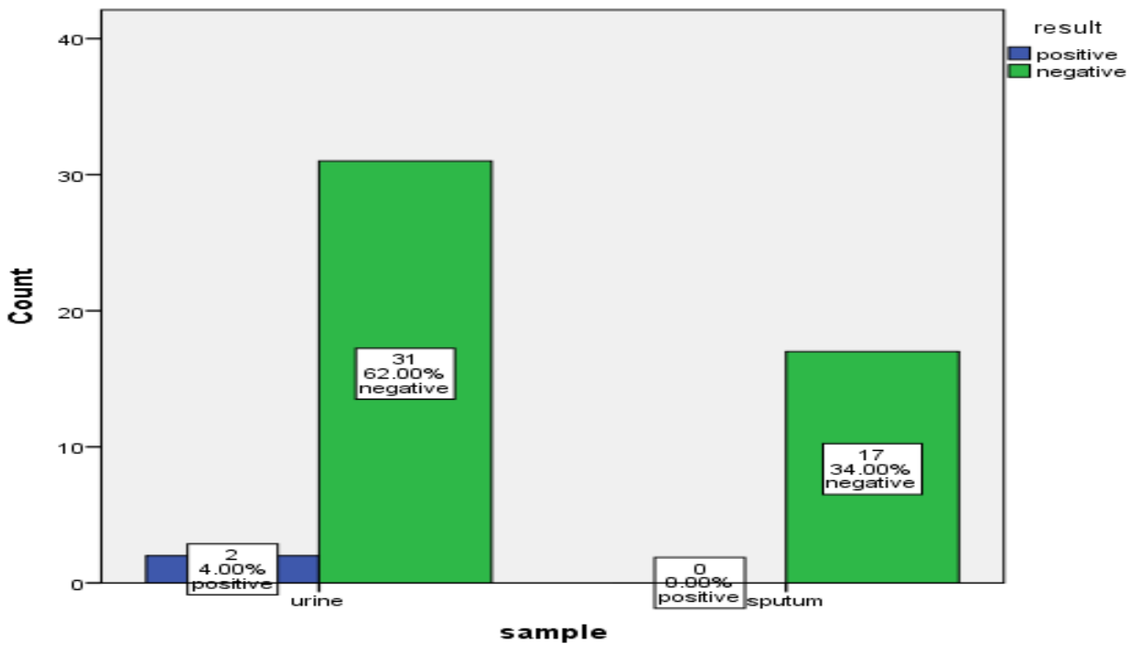
## 4.5. The Relationship between the presence of *mcr-1* gene and gender

The distribution of enrolled patients were 35 male (70%) and 15 females (30%) as shown in (Table 4-1). *mcr-1* gene were found to be 1(2%) in female, and 1(2%) in males (figure 4-1). There was no significant association between the presence of *mcr-1* genes and gender (*P* value= 0.5).

**Table (4-4):** The association between the presence of *mcr-1* gene and gender

| **Gender** | **Result** | | **Total** |
|---|---|---|---|
| | **Positive** | **Negative** | |
| Female | 1 | 14 | 15 |
| Male | 1 | 34 | 35 |
| Total | 2 | 48 | 50 |

**Figure 4-3: Distribution of positive *mcr-1* isolates among specimens source.**




## 4.6. Relationship between phenotypic, genotypic positive isolates and the specimen site.

**Table 4-5:** Relationship between phenotypic, genotypic positive isolates and the specimens' site. *P* value= 0.1.

| **Specimen sources** | **Total** | **Colistin Resistance** | ***mcr-1* Positive** |
|---|---|---|---|
| | | **Number** | **Number** |
| **Urine** | **32** | **8** | **2** |
| **Sputum** | **18** | **0** | **0** |
| **Total** | **50** | **8** | **2** |

## 4.7. Relationship between *mcr-1* Positive Isolates and Colistin Resistance

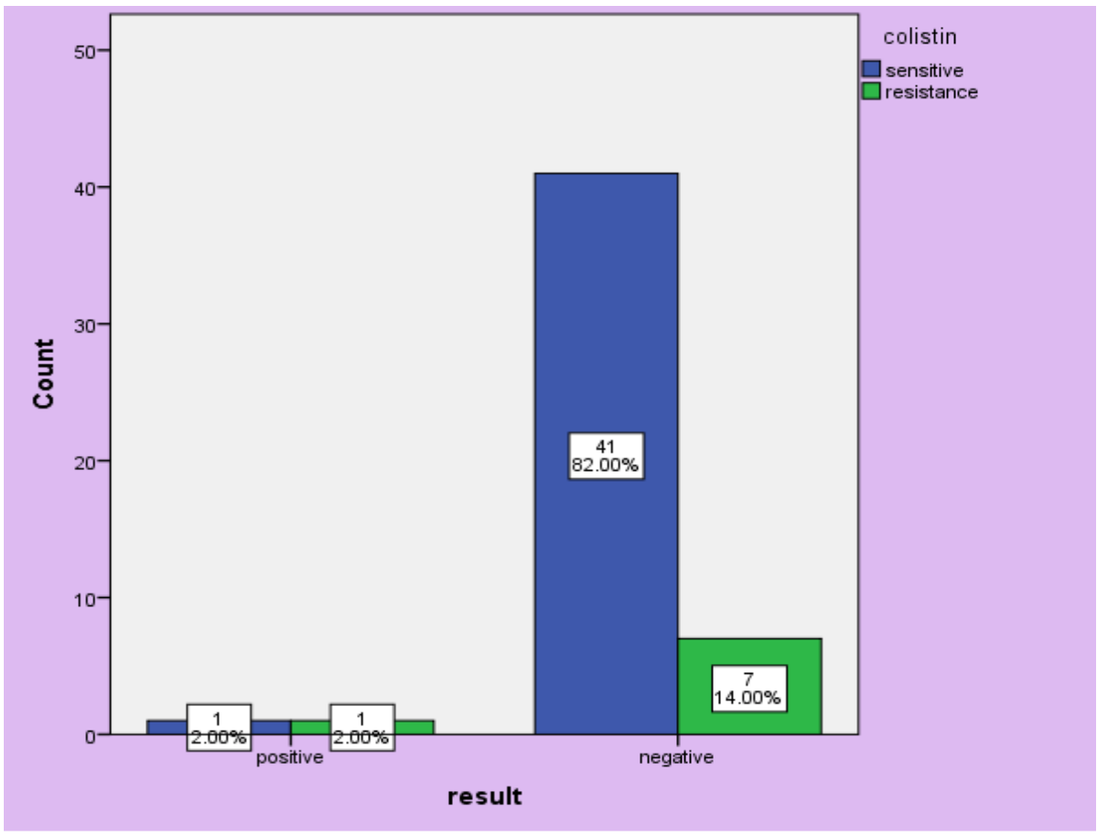



**Figure 4-4**: Relationship between *mcr-1* positive Isolates and colistin Resistance

# Chapter Five

# Discussion, Conclusion and Recommendations

## 5.1 Discussion

Antibiotic resistance is a major issue around the world. This phenomenon has led clinicians to adapt treatment strategies and to use powerful, broad spectrum antibiotics, such as Carbapenems against multi-drug resistant Gram-negative bacteria. However, the recent emergence of Carbapenemase-producing bacteria around the world (Grundmann *et al*., 2017) has obliged clinicians to turn, as a last resort, to Colistin (Biswas *et al.,* 2012**;** Stein and Raoult, 2002). The recent identification of a plasmid-encoded polymyxin resistance mechanism (*mcr-1*) among human and animal enterobacterial isolates is a source of concern.

The findings of this study demonstrate the low presence of *mcr-1* gene among *K.pneumoniae* isolates in a different area at Khartoum State, this may reveal the possible spread of pan-drugs resistance bacteria if this gene acquired by MDR organism specially Carbapenemase producing one. For the fact that *mcr-1* gene found in transferable plasmids, which can easily acquire by other bacteria from same or different species by horizontal transferred.

The result of this study showed that 2(4%) clinical isolates out of (50) have the *mcr-1*gene. Comparing with other studies, It is much higher than early detection of *mcr-1* such as in China (1.4%) from *E. coli* in 2016 and (0.2%) from Europe (Denmark) (Caniaux *et al.,* 2017). A (9) *E.coli* isolates from patients in South Africa was reported to carry *mcr-1*gene during 2014-2016 (Coetzee *et al.,* 2016), accordingly, this plasmid-encoded gene is of special concern to public health. Other study by Lin Cao and his group they realized that six (0.06%) strain carried the *mcr-1* gene from 1112 isolated *E.coli* their result differ from ours by the larger sample size and they focused in stool sample (Lin *et al*., 2018). In line with other studies conducted here in Sudan, study by (Adam *et al.,* 2017) in Sudan they report the presence of *mcr-1* gene in seven (14%) isolates of *Enterobacteriaceae*, isolated from Alzaytona and Sharg Elneel Hospitals in Khartoum State, Sudan. Also showed that *mcr-1* gene is most common in *E.coli* isolates, just one isolate of *K.pneumoniae* has shown to be *mcr-1* gene positive. The Colistin use is extremely uncommon in hospitals in Sudan, and it is probable that these isolates are present in the community, rather than arising because of selective pressure in hospitals.

Notably in this study, one of the *mcr-1* positive *K.pneumoniae* isolates, was sensitive to Colistin by disk diffusion test, however we have negative *mcr-1K.pneumoniae* isolates resist for Colistin at the same time, further investigation needed to explain this result, and determine whether *mcr-1* expression plays a role in this variability, or other theories and mechanism are involved. Most of the isolated organisms were highly resistant

to Ceftazidime (50/50), Ciprofloxacin (43/50) and Amikacin (34/50). This could be attributed to irrational use of antibiotics in Sudan (Elsiddi *et al*., 2010), which increases the selection pressure for resistance on bacteria (Elsiddi *et al.*, 2005).

The positive *mcr-1* isolates included in this study exhibited a range of antibiotic susceptibility profile. One of the positive *mcr-1* isolates were still sensitive toward Carbapenems (Imipenem and Meropenem), and the other positive *mcr-1* gene was resistant for Carbapenems. All isolates were resistant to Amoxicillin and, 3rd generation Cephalosporin (Ceftazidime), Ciprofloxacin and Aminoglycoside (Amikacin). Indicating that these highly resistant organisms are already present in our population.

The high percentage of positive isolates was recovered from urine specimens 2(4%) While sputum specimens showed no *mcr-1* has been detected.

## 5.2.Conclusion

This study confirm the presence of *mcr-1* gene among clinical isolates of *K.pneumoniae* in Khartoum state, Sudan was2 (4%). Out of (50) *K.pneumoniae* clinical isolates carried the *mcr-1* gene, also the study revealed that the genotypic positive isolates of *mcr-1* gene do not reflect phenotypic resistance to Colistin, and there were about 1(2%) *mcr-1* gene positive *K.pneumoniae* isolates still susceptible to Colistin antibiotic by disk diffusion test.

## 5.3 Recommendations

1. The government must take some steps to stop spread of resistance bacteria, especially which is positive to *mcr-1* gene.
2. Antibiotics should only be used when needed, the right drug, right dose, right route and in the right time.
3. Narrow spectrum antibiotics should be used rather than broad spectrum.
4. Culturing and sensitivity testing of pathogenic bacteria should be taken before treatment begins.
5. Health care provider should be very trained and aware about how to minimize the spread of resistant bacteria by using proper sanitation techniques includes hand washing or disinfecting between each patient.
6. Further epidemiological and interval, with larger sample size and covered wide region in Sudan to nationally assess *mcr-1* carried bacterial infection should be initiated and preventive measures to decrease the spread and transmission of this gene are warranted in Sudan.

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# APPENDICES

## APPENDIX 1

**Questionnaire**

Name: …….

Age: …….

Sex: ……

Isolated microorganisms: ……

Sensitive to:

……..……

Resistant to:

……..

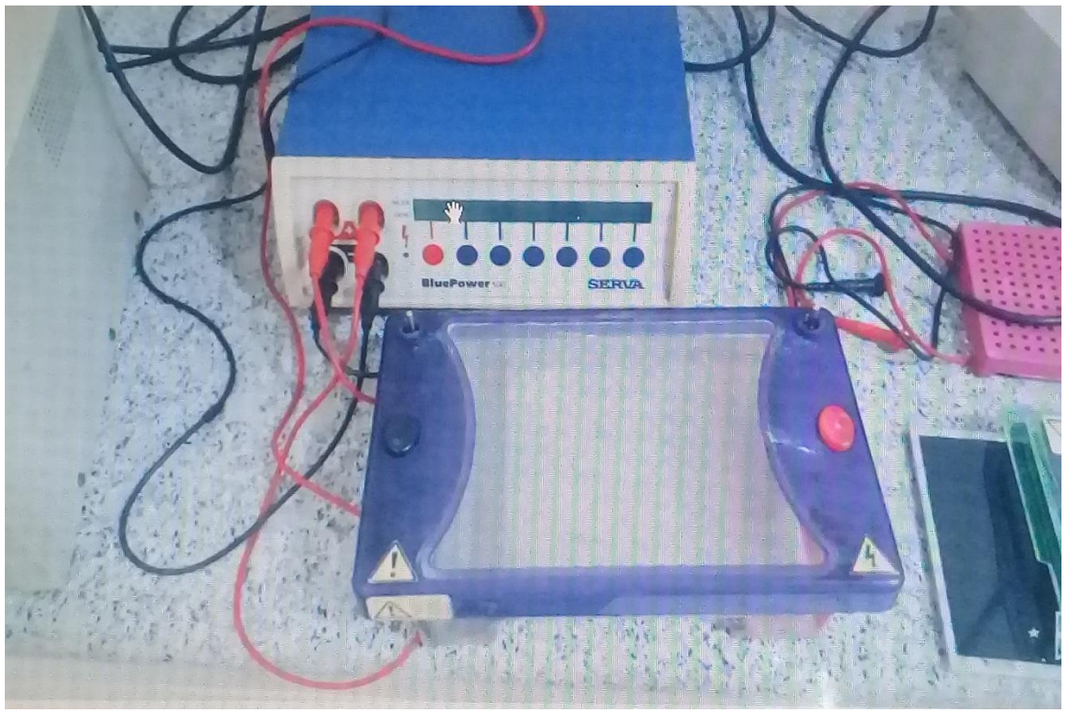
**APPENDIX 2**

**COLOURED PLATES**


**Colour plate: 1 UVitec UK (Trans-illuminator).**

**Color plate 2: Microwave.**

**Color plate 3: Thermo-cycler**




**Color plate 4: Gel electrophoresis and power supply device**