Sudan University of Science and Technology

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Molecular Detection of Plasmids Mediated Colistin Resistance (*MCR-1*) Gene Of Already Identified *Klebsiella pneumoniae* Isolated from Clinical Specimens in Khartoum State, Sudan

الكشف الجزيئي عن جين MCR-1 البلازميدي المقاوم للكولستين في الكلبسيلة الرئوية المعرفة مسبقا المعزولة من عينات سريرية من ولاية الخرطوم، السودان

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الآية

بسم الله الرحمن الرحيم

قال تعالي:

﴿ وَلَمَّا بَلَغَ أَشُدَّهُ وَاسْتَوَىٰ آتَيْنَاهُ حُكْمًا وَعِلْمًا ۚ وَكَذَٰلِكَ نَجْزِي الْمُحْسِنِينَ﴾

صدق الله العظيم

سورة القصص الآية 14

Dedication

For all those who believed in me and gave me confidence to step up and go on. Everyone who have a cruel life and keep moving in the face of difficulties.

To the all favourite places, beautiful times and little details that make sense of waking up every morning.

For those who have been deprived of education and all people who lost invaluable things toward the Sudan.

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Abstract

Antibiotic resistance is global crisis rapidly disseminated causing huge health and economic problem. Plasmid mediated colistin resistance gene (MCR-1) which has the potential to transmit horizontally between bacteria strains and specie is an important causes of colistin resistance. Fifty isolates of already identified *K.pneumoniae* were collected from several hospital at Khartoum State from Jun to September in 2019. For confirmation the isolates were cultured on Nutrient agar and Cystine lactose electrolyte deficient (CLED) agar to detect colony growth characteristics, biochemical tests were also performed, Antimicrobial susceptibility was done, using Kirby Bauer diffusion method. The following antibiotics were used: Amoxicillin, Ciprofloxacin, Amikacin, Ceftazidime, Imipenem and Colistin. DNA was extracted using boiling method and PCR was done for *K.pneumoniae and mcr-1* gene identification usig following primer (*mcr-1 F* (5' CGGTCAGTCCGTTTGTTC-3') and *mcr-1R* (5'-CTTGGTCGGTCTGTAGGG-3')). All the isolates were confirmed as *K.pneumoniae*. The isolated organisms in this study were highly resistant to amikacin (90%), ceftazidime (95%), ciprofloxacin (80%) and amoxicillin (100%). There were 20 (40%) isolates resistant to colistin and 30 (60%) were sensitive. PCR amplification showed that only 1 (2%) clinical isolates carried mcr-1 gene. The results was reported the presence of *mcr-1* gene in *K.pneumoniae* isolates.

المستخلص

تعتبر مقاومة المضادات الحيوية أزمة عالمية تنتشر بسرعة وتتسبب في مشاكل صحية واقتصادية ضخمة. إن الجين المقاوم للكوليستين بوساطة البلازميد (MCR -1) ، والذي لديه القدرة على الانتقال أفقياً بين البكتيريا وسلالات البكتيريا ، يشكل سبباً مهماً لمقاومة الكوليستين. تم جمع خمسين عينة من بكتريا الكلبسيلا المعرفة مسبقا والمعزولة من عدة مستشفيات في ولاية الخرطوم في الفترة من حزيران/يونيو إلى أيلول/سبتمبر في عام 2019، للتأكد، تتم تزريع العينات المعزولة على وسط الاجار المغذي و اجار الكليد ، للكشف عن خصائص نمو المستعمرات واختبارات الكيمياء الحيوية أيضا ، ثم تم أجراء إختبار الحساسية للمضادات من خلال استخدام المضادات الحيوية التالية: الأموكسيسيلين ، السيبروفلوكساسين ، الأميكادين ، السيفالوكساسين. تم استخراج الحمض النووي و اختبار البلمرة التسلسلي لتحديد الكلبسيلا الرئوية و *mcr -1* بإستخدام البرايمر التالي

(*mcr-1F* (5'-CGGTCAGTCCGTTTGTTC-3') and *mcr-1R* (5'-CTTGGTCGGTCTGTAGGG-3')).

تم تأكيد جميع العزلة على أنها كلبسيلا رئوية . وكانت الكائنات المعزولة في هذه الدراسة شديدة المقاومة للأميكاسين (90%) ، والسيفتازيديم (95%) ، والسيبروفلوكساسين (80%) ، والأموكسيسيلين (100%). ووجد أن هنالك 20 (40%) من العينات المعزولة مقاومة للكوليستين .

وأظهرت نتائج اختبارات البلمرة التسلسلي ان هناك 20 (40%) من العينات المعزولة مقاومة للكوليستين و30 (60%) حساسة للكوليستين .وايضا اظهر اختبار البلمرة التسلسلي أن واحد (2%) فقط من العزل السريري يحمل جين *mcr-1* .تم الإبلاغ عن نتائج وجود جين *mcr -1* في الكلبسيلا الرئوية المعزولة.

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List of Abbreviations

Abbreviations	Complete words
AAC	N-acetyltransferases
AMEs	aminoglycoside modification enzymes
BMD	Broth microdilution
CAP	Community-acquired pneumonia
CBDE	Colistin broth-disk elution
CDC	Centres of Disease Control
CDT	Combined Disc Test
CMR	Colistin MIC Reduction
CMT	Colistin MAC Test
CMS	Colistimethate
CXCL1	C-X-C motif ligand 1
DA	Dipicolinic acid
DW	Distilled water
EDTA	Ethylene diamine tetra-acetic acid
EMB	Eosin methylene blue
ESBL	Extended spectrum beta-lactmase
hv-KP	hypervirulent strains of *K. pneumonia*
ICUs	Intensive care units
IL	Interleukin

KPC	*Klebsiella pneumoniae* carbapenemase
KPC-kp	*Klebsiella pneumonia* Carbapenemase Producing *K. pneumonia*
LPS	Lipopolysaccharide
MCR	Mobile Colistin Resistance gene
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
µl	Microliter
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydride
PCR	Polymerase Chain Reaction
PEtN	Phosphoethanolamine transferases
PMCR	Plasmid mediated colistin resistance
RPNP	Rapid Polymyxin NP test
RIF	Rifampin
UTI	Urinary tract infections

1. INTRODUCTION

1.1. Introduction

Antimicrobial agent have been discovered long time ago, among this 70 year this agent save a world from infectious disease and death of patients. The wide and long use of antimicrobial lead to appearance of new bacteria with antibiotic resistance potential. Recent study made by the Centres of Disease Control and prevention(CDC) proved that there are about 2millions patients suffering from antibiotics resistance and approximately 100,000 of these patients die and $4.5 billion annually for healthcare (Chusri and Tuanyok, 2018). Failure to apply infection control measures in a hospital and outside it is one of most causes of antimicrobial resistance (Giedraitienė *et al.*, 2011). Other important reason of bacterial resistance spread is use of antimicrobial agent in non-human niches in which the bacteria acquire resistance and pass it by different method to human normal flora, as important example *Escherichia coli* ciprofloxacin resistance is associated with the use of fluoroquinolones in aviculture (Džidić *et al.*, 2008; Giedraitienė *et al.*, 2011). In other way this massive and continuous use of antibiotic can result in developing of resistance to one or more drugs which known as multidrug resistance (MDR), multi drug resistance observed in different clinical important bacteria as *Klebsiella pneumoniae* (Alekshun and Levy, 2007). *Klebsiella pneumoniae* is one of the most important hospital and acquired opportunistic pathogen can cause UTI, pneumonia and other tissue infection worldwide (Ullmann, UPodschun, 1998). Community-acquired pneumonia (CAP) commonly caused by *Klebsiella pneumoniae* in addition to 10% of all hospital acquired infections (Nordmann *et al.*, 2009). The high ability of *Klebsiella pneumoniae* to develop antibiotic resistance and causing a serious infection made

Klebsiella pneumoniae as one of important microorganism in last years (Nordmann *et al.*, 2009). In addition *Klebsiella pneumoniae* one of most cause of multidrug resistant infections worldwide for this the Recent studies highlight the emergence of multidrug resistant *Klebsiella pneumoniae* strains specially those strains showing resistance to colistin as last line of treatment (Kidd *et al.*, 2017).

Colistin, also known as polymyxin E, an antibiotic has been use for long time in veterinary medicine, but recently colistin is used in human medicine as one of important and last choice for treatment of infection caused by multi drug resistant bacteria, especially carbapenem resistant *Enterobacteriaceae* (Yang *et al.*, 2019). Even colistin is used as the last treatment option for *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* (KPC-*Kp*) but reports of colistin resistant *Klebsiella* are documented around the world (Tzouvelekis *et al.*, 2012; Olaitan *et al.*,2014). *Enterobacterales* are sensitive to polymyxins in addition to significant activity against non-fermentative Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter* spp but some Gram-negative species are naturally resistant to polymyxin: *Proteus* spp., *Morganella morganii*, *Providencia* spp. Due to last massive use of colistin, the resistance of *Klebsiella pneumoniae reach* (1.5–6.8%), in contrast of low rates in *Escherichia coli* (0.2–0.6) (Dalmolin *et al.*, 2018).

Almost all mechanism of colistin resistance are mediated by chromosomal mutations (Bell *et al.*, 2019). First report of plasmid mediated colistin resistance gene was in 2015 in china, then approximately 17 countries observe presence of *mcr-1* gene such as Denmark, Netherland, France and Thailand (Liu *et al.*, 2016)

1.2. Rationale

Antibiotic resistance has been described as one of serious crises all over world and infection with multidrug-resistant (MDR) organisms are lead to increase of mortality rather than other infection in addition to economic issue estimated by billion dollars per year and death cases which reach at least 23,000 people die annually in the USA as a result of an infection with an antibiotic-resistant organism. The high spread of *Klebsiella pneumoniae* infection and high virulent strain, recently in the European Centre for Disease Prevention and Control many of *Klebsiella pneumoniae* were resistant to one antimicrobial group at least as cephalosporins and aminoglycosides. In addition as one of MDR organisms *Klebsiella pneumoniae* identified as an urgent threat to human health by the World Health Organization, the US Centers for Disease Control and Prevention and the UK Department of Health (Kidd *et al.*, 2017). With increase spread of multi-drug resistant *Klebsiella pneumoniae* although there are increase in using of colistin antibiotic which is last treatment resort for multidrug resistant bacteria, lead to a serious arising and resistance to colistin by plasmid mediated colistin resistant gene (*mcr-1*) as one of main resistance mechanisms. Taking into consideration a high nephrotoxicity and neurotoxicity of polymyxin E, in last four years colistin resistance *Enterobacteriaceae* in general and *Klebsiella pneumoniae* in particular are marked in Sudan as the studies was observed (Nahed *et al.*, 2017). As one of rare study to detect mcr-1 gene we conduct this study to detect mcr-1 gene in colistin resistance/sensitive *Klebsiella pneumoniae.* In addition to highlighting on ability of presence of *mcr-1* gene silent dissemination (carrying the *mcr-1* gene which are susceptible to colistin) which have already described in few cases around world.

1.3. Objective

1.3.1 General objective

To detect the presence of *mcr-1* gene in clinical isolates of *Klebsiella pneumoniae* in Khartoum State.

1.3.2 Specific objectives

1. To determine the sensitivity of *K.pneumoniae* isolates from different clinical specimens towards Colistin.
2. To amplify the Colistin resistance gene MCR-1 from *K.pneumoniae* clinical isolates.
3. To determine the occurrence of plasmid-mediated colistin resistance gene (*mcr-1*) in *K.pneumoniae* clinical isolates.
4. To correlate between the presences of mcr-1 and different study variables.

2. Literature Review

2.1 Antimicrobial resistance

The Centres for Disease Control and Prevention studies show that as result of infection with an antibiotic-resistant organism there are at least 23,000 people die annually in the USA(Frieden, 2013). Antibiotic resistance is old and Anticipated mechanism use by organism when it interact with own environment by using natural molecules.

Now antibiotic resistance play an important role in many complex medical approaches such as cutting-edge surgical procedures, solid organ transplantation, and management of patients with cancer. In addition, antibiotic resistance cause nearly 300 million premature deaths by 2050, with a loss of up to $100 trillion to the global economy (Munita and Arias, 2016)

Genetically, and depends on biology of a microorganism the resistance to antibiotic can intrinsic or innate. Bacterial chromosome, plasmid, or transposons can be carry the gene responsible of antimicrobial resistance, in addition this resistance can be acquired as a chromosomal mutation (Alekshun and Levy, 2007).

A phenotype resistant to antimicrobial agents depends on various factors of a host: degree of resistance expression, capability of a microorganism to tolerate resistance mechanism, initial colonization site, and other factors. The study a prove that the resistance on plasmid spread quickly within the genus and even unrelated bacterial genera, in contrast if the resistance is associated with genes on

chromosomes, resistant microorganisms will spread more slowly (Giedraitienė *et al.*, 2011)

2.2. Mechanism of antimicrobial resistance

Drug resistance can occur by different ways classify according to the biochemical route involved in resistance include:

- Antimicrobial molecule alteration.
- Prevention of intracellular drug accumulation by cell permeability change.
- Protection, modification and/or modification of drug binding site. (Wright, 2005; Wilson, 2014)

2.2.1 Antimicrobial molecule alteration

2.2.1.1. Chemical alterations of the antibiotic

One of the most successful mechanism that use by bacteria by using mainly enzyme add to drug lead to inactivate it or destroy molecule itself , most common enzyme biochemical reaction are acetylation, phosphorylation and adenylation (Wilson, 2014). This way see mainly in case of aminoglycoside (for example, kanamycin), chloramphenicol, and β-lactams. There are large group of aminoglycoside modification enzymes (AMEs) as N-acetyltransferases (AAC) which acetylate aminoglycoside. (Walker and Walker, 1970; Benveniste and Davies, 1973).

2.2.1.2 Destruction of the antibiotic molecule

β-lactam resistance due to destruction of amide bond of β- lactam ring by action of β- lactamases enzyme is example for this mechanism . In 1940s the β- lactamases has been detected (Abraham and Chain, 1940; D'Costa *et al.*, 2011). After widely

use of penicillin some strain of *S.aureus show* plasmid-encoded penicillinase, To solve this problem and decrease susceptibility to penicillinase, new β-lactam drug with more wide range was discovered but plasmid-encoded β-lactamase against those new drug was found. More than 1,000 different β-lactamases have been described encoded in gene termed *bla* (Munita and Arias, 2016).

2.2.2 Prevention of drug penetration and cell permeability change

Cytoplasmic membrane (mainly in gram negative bacteria) is one of important target for antibiotic, therefore the bacteria use outer membrane as first line of defence to limit and decrease influx of drug substance by change of permeability, tetracycline and β-lactams are example of effected molecule, This mechanism called decreased permeability (Pagès *et al.*, 2008).

Other mechanism in this type is Efflux Pumps, this mechanism have been use depend on eject A toxic substance of drug out of bacteria cell, the *E.coli* was first bacteria show efflux pumps mechanism on 1980s. β-lactams, carbapenems and polymyxins are example of wide range of antibiotics that can be affect by this resistance mechanism which encoded by gene locate in MGEs or in the chromosome (McMurry *et al.*, 1980).

2.2.3 Change of drug binding sites

This mechanism use by bacteria to prevent interaction of antibiotic to target site. Bacteria use different way to prevent interaction which include protection, modification of target site.

2.2.3.1 Target protection

Some genes found in bacterial chromosome are encoded proteins for target protection, most of this gene are carried by MGEs. As example of drugs affected by this mechanism include tetracycline, fluoroquinolones and fusidic acid.

2.2.3.2 Target site modification

This mechanism as all other type, the final effect is about decrease affinity of drug on target site. It is affect most of antibiotic compound with differ ways include: mutation (point mutation) in the gene encoded the target site, enzymatic alteration and replacement of target site.

One of classical and clear example of mutation resistances, is the mutation in amino acid of bacterial gene (rpoB) which result in rifampin (RIF) resistance. RIF inhibiting the DNA-dependent RNA polymerase, consist of complex enzyme and β subunit, this β subunit have highly conserve structure for binding rifampin result in inhibition of transcription mechanism. This conserve area encoded by (rpoB) gene, the resistance occur as result of amino acid substitution mutation in this gene.

well-characterized example of target modification cause by enzyme is macrolides, lincosamides, and streptogramin B antibiotics resistance as result of mono- or dimethylation in their target site , this mechanism depend on methylation of high conserve region in bacteria ribosome (50S ribosomal subunit), the methylation occur in adenine residue in position A2058 of the 23rRNA (Munita and Arias, 2016).

2.3. Mechanism of *Klebsiella pneumoniae* resistance

The resistance of *K. pneumoniae* rising up in last few year, even a simple infection as UTIs can be life threating. The most two important mechanism of *K.*

pneumoniae to resist antibiotic include, the expression of extended spectrum-lactamases (ESBLs) and other mechanism is expression of carbapenemases by *K. pneumonia* (Marra *et al.*, 2006).

2.3.1. Extended Spectrum β-Lactamases (ESBLs)

ESBLs are group of enzyme produce exclusively in Gram-negative organisms were first isolated in Germany in 1983 , ESBLs are hydrolyze broad-spectrum β-lactams result in resistance to most beta-lactam antibiotics mainly as result of taken broad spectrum cephalosporins antibiotics (Marra *et al.*, 2006)

The resistance to antibiotics including penicillins, cephalosporins (cefotaxime, ceftriaxone, ceftazidime) and to monobactam (aztreonam) but are not active against cephamycins and carbapenems (Romanus *et al.*, 2009).

ESBLs are plasmid mediated encoded by transferable conjugative plasmids, ESBLs are more prevalent in *Klebsiella pneumoniae* (ranges from 5 to 25% in several parts of the world) and some other bacteria are producing it includesy *E.coli*, *Enterobacter* spp., *Salmonella* spp, *Morganella* spp*, Proteus* mirabilis, *Serratia marcescens* and *Pseudomonas* spp (Romanus *et al.*, 2009).

2.3.2. *Klebsiella pneumoniae* carbapenemase (KPC)

Carbapenemases is different classes of enzyme lead to inactivation of some antibiotic include carbapenem, cephalosporin and penicillin antibiotic, and make bacteria resistance to carbapenem antimicrobials (e.g., imipenem, meropenem). The enzyme produce by *Klebsiella pneumoniae* known as *Klebsiella pneumoniae* carbapenemase (KPC). Recent study show that *K. pneumoniae*. one of bacteria can spread antimicrobial resistant gene to other gram negative bacteria in addition of the truth, most of multidrug-resistant organisms in organism was firstly described

in *Klebsiella*, recently , multidrug-resistant and carbapenems resistant *K. pneumoniae*. Hard to treat them and the treatment restricted on combination therapy and to colistin (Holt *et al.*, 2015; Lam *et al.*, 2018). Moreover hypervirulent *K. pneumoniae* that resist to Carbapenem one of most cause of fatal nosocomial infections (Zhang *et al.*, 2016).

Some study observe ESMBLs as one of the important mechanisms associated with colistin resistance in colistin resistance co-producers is: 2.3–5.5% for *Enterobacterales* (>11.5% are *K. pneumonia*), also a high percentage found in Carbapenemase producers, 6.2–12.0% for *Enterobacterales* (14–36.6% are *K. pneumoniae*) (Dalmolin *et al.*, 2018).

2.4 *Klebsiella pneumoniae* (*K. pneumoniae*)

2.4.1 Historical background

The organism *Klebsiella* belong to family *Enterobacteriaceae* and order *Enterobacterales* which was named after a German microbiologist Edward Klebs (1834-1913) (Ryan and Ray, 2004). Friedlander's bacterium was a name of *K. pneumoniae* in 19th century which it was first isolated by Carl Friedlander from patient died due to pneumonia (Friedländer, 1882; Merino *et al.*, 1992).

2.4.2. Definition

K. pneumoniae is a Gram-negative, encapsulated, non-motile, lactose fermenting, facultative anaerobic rod shape bacterium (Siri *et al.*, 2011). There are two strains of *K. pneumoniae, non- virulent K. pneumoniae known as* classic *K. pneumoniae*" (cKp) , other one is hypervirulent strains of *K. pneumoniae* (hv-KP) (Khaertynov *et al.*, 2018).

2.4.3. Habitat

K. pneumoniae was found in environment and normal flora colonized human mucosal surface, mouth, intestine and skin without causing diseases but if it disseminated to other tissue it can causes serious infection and the most cause of both community and hospital acquired pneumonia (Dao *et al.*, 2014; Paczosa and Mecsas, 2016)

2.4.4. Mode of transmission

K. pneumoniae can spread endogenously or from person to person especially hospitalized patient. The organism can access to the body by inhalation or direct inculcation through epithelial cell surfaces (Forbes *et al.*, 2007).

2.4.5. Antigenic structure and virulence factor

Of course, this virulent factor play important role in different *K. pneumoniae* infection and strains and the most important *K. pneumoniae* virulence factor: capsule, LPS, fimbriae (type 1 and type 3).

2.4.5.1 Capsule:

The most important virulence factor of *K. pneumoniae*, It's a polysaccharide matrix coated bacteria (Lawlor *et al.*, 2006). Hypermucoviscous capsule is a type of capsule produce by HV *K. pneumoniae* strain which contain mucoviscous exopolysaccharide and play role in pathogenicity   (Yu *et al.*, 2006; Yeh *et al.*, 2007). K antigen (k1 and k2, up through k78) it's the name of capsular polysaccharide that made up both of classic capsule and hyper virulent capsule , k

antigen is strain specific polysaccharide and can be detect by serological method (Pan *et al.*, 2008). Based on some study, the *K. pneumoniae* strains consist of K2 type are most prevalent, k1 and k2 show virulent than other strain this may related to many reasons one of them is the amount induce of reactive oxygen species release from human neutrophils in which k1 and k2 induce a smaller amount of reactive oxygen species than other serotypes . The secondary thought is that k1 and k2 are resistant to phagocytosis by human immune cell than other *K. pneumoniae* strains (Paczosa and Mecsas, 2016).

2.4.5.2. Lipopolysaccharide

Also called Endotoxin, lipopolysaccharide (LPS) is One of the important cell membrane component of Gram-negative bacteria, comprised of an O antigen, a core oligosaccharide, and lipid A which encoded by genes in the wb, waa, and lpx gene clusters, respectively (Raetz *et al.*, 2009; De Majumdar *et al.*, 2015). Even that LPS is an important virulence factor but also can activate immune system. Lipid A, portion of bacteria LPS can be detected by pattern recognition receptor as TLR4, stimulation of this receptor lead to activate neutrophil and macrophage throw cytokines and chemokines production (Paczosa and Mecsas, 2016).

2.4.5.3. Type 1 and 3 Fimbriae

It is an adhesive structure play important role, *K. pneumoniae* virulence factor and pathogenicity factor. Recently other adhesive structure have been discovered on surface of *K. pneumoniae* as KPF-28 and capsule-like material (Favre-Bonte *et al.*, 1995; Di Martino *et al.*, 1996). Type 1 fimbriae are thin, thread-like structure

express on cell surface of 90% of *K. pneumoniae* (Klemm and Schembri, 2000; Stahlhut *et al.*, 2009).

2.4.5.4. Siderophores:

Iron is a metal require by *K. pneumoniae* for infection process, in host cell there are irons but bound by iron transport molecules as transferrin so it difficult to obtain require amount for microorganism. Therefore, like other many organism *K. pneumoniae* must obtain iron from host cell, this done throw secretion of Siderophores. Siderophores is iron chelating molecules with high affinity to iron than host transport protein. To optimize colonization in different tissue and avoid neutralization of single Siderophore by host *K. pneumoniae* produce more than one Siderophores including enterobactin, yersiniabactin, salmochelin, and aerobactin. The aerobactin show the lowest affinity to iron and enterobactin is a highest one (Paczosa and Mecsas, 2016).

2.4.6. Extracellular product

K. pneumoniae produce polysaccharide and pullulanase enzyme, the last is lipoprotein in nature, work as starch debranching enzyme that released from outer membrane to growth media (Chapon and Raibaud, 1985) Also *K. pneumoniae* can mediate biofilm formation on extracellular matrix (Jagnow and Clegg, 2003).

2.4.7. *K. pneumoniae* infection

K. pneumoniae is one of the most infectious bacteria that cause severe infection mainly due to genetic change in strains lead to either hyper-virulent or antibiotic resistant (Friedländer, 1882; Merino *et al.*, 1992). It can spared and infect different site in the body causing threat life pneumonia, UTI, bacteraemia and liver abscess, the study show that hyper virulent strain cause community-acquired and

systemic infections (Taiwan and Southeast Asia) which characterize by high spread, morbidity and mortality , and classical strains of *K. pneumoniae* which cause nosocomial infections or UTIs (worldwide). (41, 48–50) (Paczosa and Mecsas, 2016)**.** Wide range of infection can cause by classic *K. pneumoniae* from pneumonia and UTIs to a serious infection such as bacteraemia (primary or secondary), the spread of primary bacteraemia in lung and bladder can cause a secondary infection (Zarkotou *et al.*, 2011; Qureshi *et al.*, 2012). The study show that *K. pneumoniae* is the secondary causative agent of bacteraemia (community-associated and nosocomial) after E.coli in all gram-negative bacteria (Ullmann, UPodschun, 1998). In addition to bacteraemia, *K. pneumoniae* is one of the frequent causes of UTIs behind E.coli, usually this UTIs can be nosocomial UTIs (2 to 6%) or community acquired UTIs   (4.3% to 7%) (Paczosa and Mecsas, 2016).

Classic type of *K. pneumoniae* cause pneumonia and urinary tract infection in addition to nosocomial infections and neonatal sepsis in immunocompromised patients. Other hypervirulent strain (hvKp) cause abscesses, meningitis, and endophthalmitis (Khaertynov *et al.*, 2018).

2.4.8. Host defences

Many of in vivo and in vitro studies are help to understand the immune defence to controlling *K. pneumoniae* infection. One of the important defence on respiratory tract is mechanical mucociliary elevator, in which microbe or pathogen trap by mucus and then shuttles it out using cilia. In genitourinary tract both of strong mechanical flow of urine (mechanical) and low pH of urine (chemical) are used to prevent *K. pneumoniae* or other bacteria from reaching bladder. This Pathogen face physical and chemical defences in GI tract , and as respiratory tract mucus prevent

organism binding to epithelium , while bile and digestive enzyme create hard chemically environment (Paczosa and Mecsas, 2016).

If *K. pneumoniae* pass this initial barriers the humral and cellular mechanism are overcome. An example of humral mechanism include the complement system, the activation of complement system mediate cell lysis after forming pores by membrane attack complexes, in addition to release of proinflammatory mediators. Other humoral mechanism include defensins which are bactericidal factors found in lung, and transferrin as bacteriostatic factors. Some studies show *K. pneumoniae* that can protect against many humoral defence, in addition to susceptibility of *K. pneumoniae* variable from strain to strain (Paczosa and Mecsas, 2016). As example human beta defensin 3 (HBD-3) is more efficient to killing *K. pneumoniae* than HBD-1 and HBD-2 (Moranta *et al.*, 2010).

Innate cellular defence also play an important role mainly in lung in which alveolar macrophage work as phagocytic cell in addition to production of chemokines and cytokines which lead to amplification of immune response. After infection this cytokines produced by macrophage (interleukin-8 (IL-8), CXCL1, complement, and leukotriene B4) attract neutrophil to the infected tissue for killing and clearance of bacteria (Paczosa and Mecsas, 2016).

2.4.9. Mortality and morbidity

Klebsiella cause a serious infections with high mortality rate, as example k.pnemonia has high mortality rate reach 50% even if the patient under therapy. Mortality and morbidity is approximately 100% in alcoholism and septicaemia (Ashurst and Dawson, 2020).

2.4.10. Epidemiology

K. pneumoniae found in 5% to 38% of community, individually, k.pnemoniae carry in small amount in stool and nasopharynx, this number increase in hospitalized patients in comparing within community. In addition the Pneumonia caused by *K. pneumoniae* are tow type either community-acquired or hospital-acquired pneumonia, the study was showed that approximately 11.8% of hospital-acquired pneumonia case by *K. pneumoniae* , overall 3% to 5% of community-acquired pneumonia cause by *K. pneumoniae* but those number may reach 15% in case of developing countries such as Africa (Ashurst and Dawson, 2020)

2.4.11 Treatment / Management

After confirm of *K. pneumonia* infection the suitable treatment should be follow. In case of community-acquired *K. pneumoniae* pneumonia, as monotherapy either a third or fourth generation cephalosporin given in a 14-day of treatment or a respiratory quinolone. Although aztreonam or a respiratory quinolone is the suitable therapy in case of patient have allergic against penicillin. In addition a monotherapy of carbapenem antibiotic is require for nosocomial infections also in case of ESBL infection. When carbapenem-resistant Enterobacteriaceae is diagnosed other antibiotics should be obtained such as: colistin (polymyxins), tigecycline, fosfomycin, and aminoglycosides (Ashurst and Dawson, 2020)

2.5. Colistin

Also known as polymyxin E, polymyxins are group of antibiotic (polymyxin A-E) different chemically but only polymyxin B and polymyxin E (colistin) has been use clinically (Falagas *et al.*, 2005).

Colistin was discovered in 1947 from a soil bacteria *Paenibacillus polymyxa* subsp. Colistinus but has been ready to use in 1959 against Gram-negative bacteria (Dalmolin *et al.*, 2018).

Firstly colistin was produced in form of inactive prodrug colistin methanesulfonate (CMS). It was one of the most antimicrobial agent with significant action against Gram-negative bacteria, notably *Pseudomonas aeruginosa*. However polymyxins show high nephrotoxicity and neurotoxicity which lead to replaced polymyxins by aminoglycosides in the 1970s.Nephrotoxicity occur within first day of therapy and sign may continue to 1-2 weeks (Li *et al.*, 2005). But When the use of a B-lactam, aminoglycoside, or quinolone is ineffective colistin has been one of limited option and use as treatment of infection cause by multidrug resistance(MDR) bacteria in particular *P.aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* (Nation and Li, 2009).

The use of polymyxin increasing worldwide which result in increase the percentage of resistance between bacteria , ther colistin resistance in *Enterobacterales* (with no intrinsic resistance) is around 0.67–1.6%, with *Escherichia coli* (0.2–0.6%), and a moderate rates in *Klebsiella pneumoniae* (1.5–6.8%), and much higher rates in *Enterobacter* spp. (13.9–20.1%) (Dalmolin *et al.*, 2018).

2.5.1 Antibacterial activity and resistance

2.5.1.1 Mode of action

The mechanism of colistin action occur in bacteria cell membrane, the characteristic structure of Gram-negative bacteria and contain LPS. The disturbance of cell membrane occur as result of electrostatic interaction between colistin (cationic) polypeptide and lipopolysaccharide (anionic) molecules of

bacteria outer membrane. The magnesium (Mg+2) and calcium (Ca+2) which are woke as LPS stabilizer can be replaced by colistin, disturbance occur locally , permeability increase result in sing out of cell content and cell death. Possible ability of polymyxins to inhibit respiratory enzyme in bacteria membrane as (type II NADH-quinone oxidoreductases [NDH-2]) is consider as other possible mechanisms of action described for polymyxins (Falagas *et al.*, 2005).

2.5.1.2 Spectrum of activity

The colistin show narrow spectrum, commonly against Gram-negative bacteria. Colistin show bactericidal activity against most members of the Enterobacteriaceae family includes *E. coli, Enterobacter, Salmonella, Shigella and Klebsiella*, and important nonfermentative Gram-negative bacteria, *P. Aeruginosa* and *Acinetobacter species* (Li *et al.*, 2005; Bialvaei and Samadi, 2015). The eukaryotic microbes and mammalian cells, *Proteus* species, *Neisseria* species, Serratia species are resistant. Gram-positive cocci and Gram-positive bacilli show no response to colistin (Bialvaei and Samadi Kafil, 2015).

Other study report colistin activity agenist *mycobacterial species*, including *Mycobacterium xenopi, Mycobacterium intracellulare, Mycobacterium tuberculosis, Mycobacterium fortuitum, Mycobacterium phlei, and Mycobacterium smegmatis* (Falagas *et al.*, 2005).

2.5.1.3 Use in human medicine

Colistin as well as colistimethate (CMS) the inactive prodrug of colistin was used mainly for treatment the Gramnegative bacterial infection. In 1950s colistin was used in Europe and Japan, then in 1959s was used in U.S after approval by the U.S. FDA. But few years later after scientist discover the high toxicity of colistins drug

especially nephrotoxicity, they were replaced with other less toxic antibiotic such as aminoglycosides, quinolones. However the colistin use was restricted to ophthalmic and topical uses with systemic use only for cystic fibrosis patients. But the systemic polymyxin are reused and considering as few, sometimes the only therapeutic option after the increasing infection by multidrug-resistant (MDR) bacteria as *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* (Poirel *et al.*, 2017).

2.5.2 Toxicity

Resently, the toxicity of CMS has been more lower due to good monitoring in intensive care units (ICUs) and fewer chemical that impurities in CMS. The two main toxic effect cause by colistin are neurotoxicity and nephrotoxicity, both of them are dose depend, the neurotoxicity is less common comparing with nephrotoxicity and reversible. Most neurotoxicity may cause peripheral and facial paresthesia, weakness, visual disturbances, confusion, ataxia, and neuromuscular blockade and with no report of neuromuscular side effect. Nephrotoxicity is more common and faster in appearance of effect which may occurring within the first week of treatment. There are factor may increase the risk of colistin nephrotoxicity, this risk factor may include: increases colistin concentrations in blood plasma above than 2.5 to 3 microgram per milliliter, coadministration of antibiotic (aminoglycoside and vancomycin) or anti Inflammatory drugs with nephrotoxic effect, other risk factor is risk related to patient (male sex, hypoalbuminemia, hyperbilirubinemia, chronic kidney disease, severity of illness and old age). Some recent study approve that CMS/colistin showed high nephrotoxicity than polymyxin B (Poirel *et al.*, 2017).

2.5.3 Mechanism of resistance

Mainly colistin resistant case is uncommon but recently several species of Gram-negative bacteria have been resistant colistin such as *A.baumannii, K. pneumoniae*

and *P.aeruginosa*. There are poor data and study about mechanism of colistin resistance, but since colistin activity against cell membrane most study suggested the resistant mechanism occur in LPS, this may include efflux pump system, modification of cell membrane and increase production of capsule (Bialvaei and Samadi, 2015).

However many of studies the mechanism is not completely clear, but resistance to polymyxin E and polymyxin B is associated with remodelling in the lipid A (Olaitan *et al.*, 2014). Several recent studies highlight the emergence of colistin resistance in MDR *K. pneumoniae* take place in lipopolysaccharide and arising from loss of function mutations of the *mgrB* gene. The *mgrB* gene is negative regulator of the *PhoQ/PhoP* signalling system, the PhoPQ two component system both of then reduce interaction of cell wall and colistin due to decrease anionic of lipid A (Cannatelli *et al.*, 2014; Kidd *et al.*, 2017). The PhoPQ signalling system also known as regulator of envelope remodelling, chiefly the lipopolysaccharide (LPS) lipid A section, and contributes to bacterial resistance to innate immune killing (Groisman, 2001; Beceiro *et al.*, 2011).

2.6. Mobile Colistin Resistance Gene 1 (MCR-1)

For long time chromosomal mutations has involved widely in polymyxin resistance without reporting of horizontal gene transfer, but in last few year, a study on *Escherichia coli* from food animals in China. The surveillance observe a highly increase of colistin resistance, in addition the study noticed colistin resistance that could be transferred to another strain. Which plasmid-mediated polymyxin resistance was predictable, Not longer, the first plasmid-mediated polymyxin resistance mechanism, *mcr1*, in *Enterobacteriaceae* has reported(Liu *et al.*, 2016).

The horizontal gene transfer is a transfer of resistance genes from one bacterium to another, this mechanism include plasmid transfer, in addition to other mechanism include transfer by viral delivery, and transfer of free DNA. This transfer occur by main three way include: transduction, conjugation (via plasmids) and transformation (Giedraitienė *et al.*, 2011). The plasmid encode up to 10% of the host cell chromosome (Alekshun and Levy, 2007). Many study show that the transfer of resistance genes is more effective than chromosomal mutation which the MDR genes are located in a DNA sequence thence in plasmid which can transferred from one plasmid to another (Hawkey, 1998).

Five year ago in china, Liu and colleagues described the transferable colistin resistance in animals and humans isolates of *E. coli* and *K. pneumonia*, this transfer mediated by which called mobile colistin resistance gene (mcr1). MCR-1 protein is a member of transferase family known as phosphorethanolamine, this gene locate in plasmid (Dalmolin *et al.*, 2018). Which decrease affinity of colistin to bacteria membrane by adding a phosphoethanolamine group to lipid A (Poirel *et al.*, 2017).

Since the first report of first plasmid-encoded colistin resistance gene *mcr*-1 the gene it has been report in other different region Asia, North Africa, Europe and North and South America. Microorganisms with *mcr-1* gene have been isolated from different source include livestock, food, environment and from infected human as well as asymptomatic human carriers (international travelers). Most report of *mcr-1* gene has been in several species of *Enterobacterales* with high rate in *E. coli*, *Salmonella*, *Shigella*, *Klebsiella*, *Vibrio* occurrence are detected and *Enterobacter* was also sporadically reported (Irrgang *et al.*, 2016).

2.6.1 Epidemiology of Plasmid-mediated Colistin Resistance

Recently, the mcr-1 was reported worldwide in Asia, Africa, Europe and America. Even that mcr-1 gene was identified in human from 2008 in *Shigella sonnei* from Vietnam and some year ago in china, but Some studies have found this gene was existed for long time and remained undetectable which there are different other studies observe mcr-1 gene in china from 1980s then it have been reported worldwide mostly in *E. coli* but other bacteria as *Salmonella, Klebsiella, Shigella, Vibrio* showed interspecies transfer of gene.This gene was detected in animals as pigs and chicken which conceder as *E.coli* reservoirs, the mcr-1 gene detected in human from different clinical sample, however the dissemination of this gene from animal to human is serious condition (Irrgang *et al.*, 2016).

Although, eight plasmid mediated colistin resistant gene variants have been described from *mcr-1* to *mcr-8* from different country and animal and some of has variant as *mcr-1, mcr-2, mcr-3, mcr-4, mcr-5*. There are 13 *mcr-1* subgroup were detected world wild differ in one nucleotide include: *mcr*-1.2 (*K. pneumoniae* from Italy), *mcr*-1.3 (*E. coli* from China), *mcr*-1.4 (*E. coli* from China), *mcr*-1.5 (*E. coli* from Argentina), *mcr*-1.6 (*Salmonella typhimurium* from China) , *mcr-1.7* (*E. coli* from China), *mcr-1.8* (*E. coli* from Brunei), *mcr-1.9* (*E. coli* from China), *mcr-1.10* (*Moraxella* spp. from Great Britain), *mcr-1.11* (KY853650.1), *mcr-1.12* (LC337668.1) and *mcr-1.13* (*E.coli* from Italy) (Dalmolin *et al.*, 2018).

2.6.2 Detection of MCR

The reference test for susceptibility profile of polymyxins is Broth microdilution (BMD), BMD is conceder as gold standard method for determination colistin MIC (Osei Sekyere, 2019). Otherwise the molecular method are consider as reference test to detect *mcr* gene and variation. The detection of MCR producing bacteria from culture is performed by few phenotypic tests the Colistin MAC Test (CMT)59; Combined DiscTest (CDT); Colistin MIC Reduction (CMR); Modified

Rapid Polymxin NP Test (modified-RPNP) and alteration of Zeta potential tests (Dalmolin *et al.*, 2018). Colistin broth-disk elution (CBDE) method on of the simple test apply for colistin antimicrobial susceptibility testing in addition to detection of plasmid mediated colistin resistanc (PMCR) genes (Bell *et al.*, 2019).

The enzymes catalytic domain of MCR is resemble that of zinc metalloproteins, absence of zinc can inhibit the enzymatic activity of MCR-1, depend on this theory there are many different type of assays use colistin MIC reduction in presence of dipicolinic acid (DA) or ethylene diamine tetra-acetic acid (EDTA) to evaluate MCR producing bacteria. Both of dipicolinic acid (DA) or ethylene diamine tetra-acetic acid (EDTA) are able to chelate zinc which is necessary to activate PEtN transferase enzymes, inhibition if PEtN transferase enzymatic activity result in inhibition of enzymatic activity of MCR-1 and colistin resistance reduction (Dalmolin *et al.*, 2018).

2.6.2.1. Colistin Mac Test (CMT)

Method for detect MCR producer, is one of broth microdilution method depend on colistin MIC increasing or decreasing in presence of zinc chelate substance. Fixed concentration of DA (900 μg/mL) was tested with colistin MIC (0.125 to 8.0 μg/mL). Absence of mcr result in MIC reduction of ≤2-fold (*mcr*-negative), in contrast to MIC increases ≥8-fold is indicate presence of mcr (*mcr*-positive) (Coppi *et al.*, 2018; Dalmolin *et al.*, 2018).

Other tests depend on EDTA was appraised by Esposito and there college include:

2.6.2.2 Combined Disc Test (CDT)

CDT assay depend on comparing size of inhibition zone between colistin (10μg) and colistin plus EDTA (100mM), 100 mM EDTA was chosen which is inhibit MCR-1 without bacterial growth. Positive result interpreted if inhibition zone around 10 μg of colistin–100 mM EDTA is increase ≥3 mm in the size comparison

to the inhibition zones of colistin without EDTA.The sensitivity of CDT were 96.7 and specificity were 89.6% (Esposito *et al.*, 2017).

2.6.2.3 Colistin Mic Reduction (CMR)

The fixed concentration used in CMR assay was 80 μg/ml, wells containing 0.06–32μg/ml of colistin without EDTA and wells containing 0.06–32μg/ml of colistin with EDTA(80 μg/ml,) was tested . MCR-1-positive reported when ≥4-fold reduction of colistin MIC containing EDTA is occur. The CMR present sensitivity and specificity of 96.7 and 83.3%, respectively (Esposito *et al.*, 2017; Dalmolin *et al.*, 2018).

2.6.2.4 Rapid Polymyxin NP test (RPNP)

RPNP test one of cheaper and easier colistin-resistant and *mcr*-producing detection assay designed by Nordmann *et al.,* (2009) (Osei Sekyere, 2019). the test depend on bacterial growth on glucose based media, color change from orange to yellow occur due to pH alteration as result of bacterial growth. A microtiter plate was used , four of wells contain colistin-free solutions, other four well contain colistin and rapid polymyxin solution, 0.85% NaCl, colistin-susceptible negative-control strain, (colistin-resistant positive-control strain (A3 and B3)), and the test isolate to first, second, third, fourth wells respectively. Positive result reported as color change from orange to yellow. However, RPNP test report higher sensitivity and specificity comparing with other test we mention, the sensitivity and specificity are 96.7% and 100.0%, respectively ((Dalmolin *et al.*, 2018; Osei Sekyere, 2019). Noteworthy, acidity of culture media where are colonies obtain can result in false-positive results such as McConkey agar, the accurate result is obtain from Mueller–Hinton agar, chocolate agar, and eosin methylene blue (EMB) agar (Osei Sekyere, 2019).

However, the reference test for detection bacterial mcr gene are Polymerase Chain Reaction (PCR) (detect only known mcr genes) and Whole Genome Sequence (WGS) (detect known or unknown colistin resistance genes). Although microarray can use for *mcr-1/-2* genes (Dalmolin *et al.*, 2018). First mcr-1 genes detected by self-designed primers using PCR was in 2016 by Nijhuis *et al.*, not so long follow by designed a quantitative Taqman® PCR, Both of are with 100% sensitive and specific (Chabou *et al.*, 2016; Nijhuis *et al.*, 2016).

Moreover, studies report that *K pneumonia is less* efficient with MCR detecting assays based on chelator substance in comparing with *E.coli* (Osei Sekyere, 2019).

Materials and Methods

3.1 Study design

This study was a cross-sectional hospitals based study.

3.2 Study area and duration

The work was carried out in Khartoum state, Sudan. The laboratory works performed at research laboratory in Sudan University of sciences and technology, during the period from February to November, 2019.

3.3 Study population

K.pneumoniae clinical isolates were obtained from Military hospital, police university hospital, royal care hospital.

3.4 Ethical consideration

Permission to carry out this study was taken from the College Of Medical Laboratory Science, Sudan University for Sciences and Technology and authorities of these hospital.

3.5 Data collections:

Data were collected from hospital records.

3.6 Sample size

A total of 50 already identified *K. pneumonia* isolates from various clinical specimens: swab, blood and catheter.

3.7 Nutrient agar

Basic medium (HIMEDIA, India)) was used for purification and support the growth of bacteria.

3.8 Gram stain

Abacterial colony was suspended in normal saline, smeared, left to dry, fixed with heat by passing slide three times over flamed heat and then stained with crystal violet stain(see appendix 2) for 30–60 seconds,

Washed, covered with Lugol's iodine (see appendix 2) for 30–60 seconds, Decolorize rapidly (few seconds) with acetone–alcohol and washed and finally covered with neutral red stain for 2 minutes, washed and dried. The dried smear examined microscopically by oil immersion (Madigan *et al.,* 2004).

3.9 Biochemical identification

The tests was carried out on kliger iron agar, tryptophan peptone water, Simmons's citrate agar and Christensen's urea agar and results was reported. All tests have been done according to standard laboratory procedures (Cheesbrough, 2006).

3.9.1 Kligler Iron agar

Kligler Iron Agar medium (HIMEDIA, India) (Appendix 3), was prepared according to the instructions of manufacturer. The sterilized straight wire was used for inoculation the butt was stabbed and then the slope streaked in a zig-zag pattern. Observation for color changes, gas and H2S production was done after 24 hours of incubation at 37oC. A yellow slope and a yellow butt indicate the fermentation of lactose and possibly glucose, this occurs in bacteria such as *E. coli* and *K. pneumoniae*. A yellow butt (acid production) and red-pink slope indicate the fermentation of glucose only, as in *Proteus spp and Pseudomonas spp*. gas production from glucose fermentation was observed as cracks and bubbles in the medium, this was showed in *K.pneumoniae* (Cheesbrough, 2006).

3.9.2 Indole test

Tryptophan peptone water (HIMEDIA, India) was used. The test organism was inoculated in a bijou bottle containing about 3 ml of sterile tryptone water (Appendix 2), and sealed well then test tube was incubated under a septic condition at 37˚C for up to 48h. Production of indole was checked by addition of 0.5 ml of Kovac's reagent, examined for a red color in the surface layer within 10 minutes

after shaked gently which indicate positive result. Positive result was observed in most strains of *E. coli*, *P. vulgaris*, *P.rettgeri*, *M. morganii*, and *Providencia* species. Other *Enterobacteriaceae* are indole negative (no color change) (Cheesbrough, 2006).

3.9.3 Citrate test

The tested organisms were suspended in a normal saline and cultured in a Simmon's citrate agar (HIMEDIA, India) (Appendix 3). By using a sterile straight wire, firstly the slope was streaked and then butt was stabbed and incubated over night at 37oC. This test was used to assist in differentiation of *Enterobacteriaceae* spp. *K. pneumonia* and some strains of *Proteus spp.*gave positive result (bright blue). *E. coli* is citrate negative (no color changes) (Cheesbrough, 2006).

3.9.4 Urease test

A medium (HIMEDIA, India) which contains urea and phenol red as indicator was used for test organism culture in (Appendix 3), by using a sterile straight wire and under a septic condition, the media was inoculated and incubated overnight at 37oC. This test was used to differentiate

Enterobacteriaceae spp. Positive result observed as production of pink color as seen in *Proteus* strains (strong Urease producers), *K.pneumoniae* (slow). And *E. coli* is Urease negative (Cheesbrough, 2006).

3.10 Antimicrobial susceptibility test

All identified isolates were subjected to antibiotic susceptibility testing discs by Kirby-Bauer diffusion method according to Podschun and Ullmann (Ullmann, UPodschun, 1998). Muller-Hinton agar (HIMEDIA, India) was prepared according to lab direction. Suspension from tested organism was prepared by emulsified few colonies in sterile normal saline and compared with turbidity of 0.5 McFarland standard. Cotton swab was used for inoculate suspension on Muller-Hinton agar

surface after dipped into standardized suspension and squeezed genteelly to remove excess from swab. Under aseptic condition and by using sterile forceps the following antimicrobial disc (HI-MEDIA, India) were applied on the surface of the agar Amoxicillin (25μg), Ciprofloxacin (5μg), Amikacin (10μg), Ceftazidime (30μg), Imipenem (10μg) and Colistin (10μg), the distance performed was 24mm between discs and 10 mm between discs and plate edges. The plate was incubated aerobically in incubator at 37°C for overnight. The plate was examined and ruler was used to measure zone diameter in (mm). The recorded zones were compared with those in the chart; results with specific organism reported as resistant (R) or sensitive (S) (CLSI, 2012).

3.10 Detection of *MCR-1* by Polymerase chain reaction

3.10.1. DNA extraction

Boiling method was used for extracting DNA. The (3-5) colonies from tested organisms were dissolved in 1ml of sterile distilled water, boiled for 10 minutes in a water bath, and then were centrifuged for 5minutes at 1000 rpm (Dashti *et al.*, 2009).

3.10.2. Primer:

Primers Sequences used for the Amplification of Gene are mcr-1F (5' CGGTCAGTCCGTTTGTTC-3') and mcr-1R (5'-CTTGGTCGGTCTGTAGGG-3'), was used for screening as previously described (Liu *et al.,* 2016).

3.10.2.1 Stock primer

Two hundred fifty μl of sterile DW was added to each vial of primer then vortex was performed.

3.10.2.2 Working Primer

Ninety μl of distilled water was added to (10 μl) of stoke primer for dissolveing and stored at (-20 ℃).

3.103. Master Mix

Master Mix kits (INtRON Maxime PCR PreMix, Korea) ready to use solution containing all reagents required were used. Storage was carried out at -20˚C.

3.11. Reaction Mixture

PCR was carried out in a 13 μl volume using the Maxime PCR PreMix kit (iNtRON Biotechnology, Seongnam, Korea) which used for detection of *MCR-1* gene in the following volumes in 0.2 ml eppenddorf tube. 5 μl of Master mix premix (i-Taq), 5 μl Deionized sterile water, 0.5 μl of each forward and reverse primer and 2 μl of DNA template.

3.12. Protocol used for amplification of the *MCR-1* gene

The amplification was done by using thermocycler PCR (SensoQuest, Germany). The PCR mixture was subjected to initial denaturation step at 94°C for 5-min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min, followed by step of extension at 72°C for 1 min and the final elongation at 72°C for 10 min.

3.13 Gel Electrophoresis

3.13.1 Preparation of Agarose Gel

Amount of 1.5 g of agarose powder (BIOLINE, UK) was dissolved in (100 ml) 1X TBE buffer (Sigma, Japan) in microwave for 2 min for heating. The mixture was cooled, 2 μl of (20mg/ml) ethidium bromide was added. Taped up the casting tray appropriately and equipped with suitable comb to form well in place then split the

mixuer on. Bubbles were removed and then gel were setting at room temp to solidify. All casting tray equipments were remove gently.

3.13.2 Electrophoresis and Visualization of PCR product

The 1x TBE buffer (Sigma, Japan) was use to flooded gel casting tray until gel surface was covered, 2µl of 100bp DNA ladder (marker) (INtRON) ladder was added to the first well of casting tray and for each run, then 4 µl of PCR products of each samples was put into each well. The gel electrophoresis apparatus was run at 100 v for 15 min (CONSORT E865, Belgium). The gel was removed and visualized under the ultraviolet Transilluminator (UVitec UK)

3.13.3 Preparation of 10X TBE buffer

Amount of 108 grams of Tris base was added to 55g of boric acid and 40 ml of 0.5 EDTA, and then dissolved into 1 liter Deionized water pH 8.0.

3.13.4 Preparation of 1X TBE buffer

Ten ml of 1X were added to 90 ml deionized water and stirring until completely dissolved.

3.14 Data analysis

Data of research was recorded and analyzed by SPSS statistical analysis software version 16.0. Using Chi-Square test for analysis and estimate p value.

4.1 Detection of MCR-1 gene among *K.pneumoniae* isolates

The isolated organisms were 50 of *K.pneumoniae,* PCR result showed that 1 (2%) clinical isolates carried *mcr-1* gene as shown on figure (4.1).
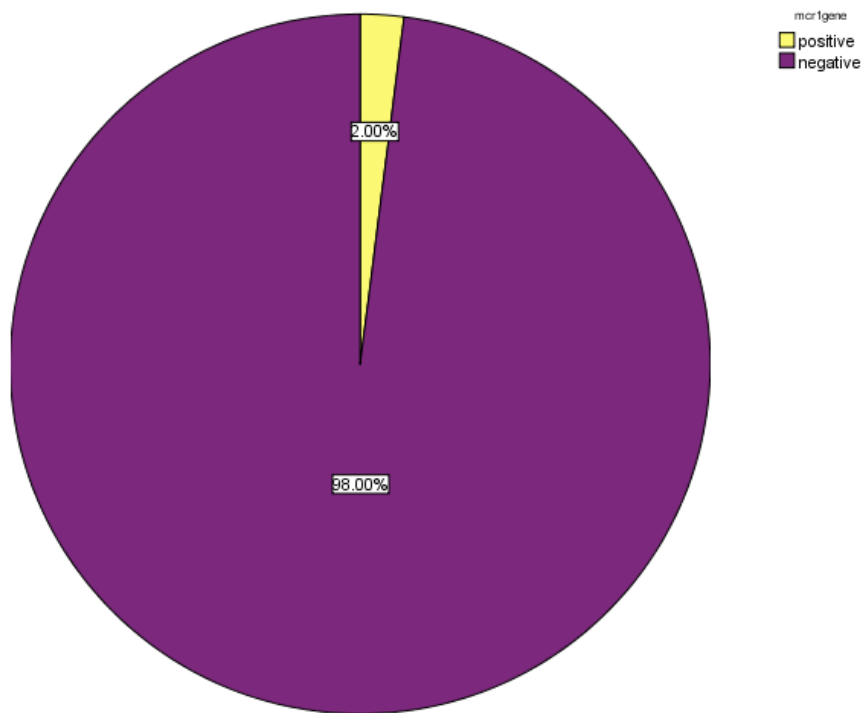
Figure 4.1: Frequency of MCR-1gene Positive K.pneumoniae isolates.

4.2 Enrolled patients

The distribution of enrolled patients from different age group were 30 males (60%) and 20 females (40%) (Table 1).

Table (1) Relationship between *mcr-1 gene* and gender.

			mcr1gene		Total	P-value
			positive	negative		
Sex	male	Count	0	30	30	
		% of Total	.0%	60.0%	60.0%	
	female	Count	1	19	20	
		% of Total	2.0%	38.0%	40.0%	0.216
Total		Count	1	49	50	
		% of Total	2.0%	98.0%	100.0%	

4.3 Distribution of mcr-1 gene among age group

The mcr-1 gene was only detected in group 71-90 infected with *K.pneumoniae*. Statically there was significant association between age of patients and the presence of *mcr-1* gene (p <0.05).

Table (2): Relationship between the presence *mcr-1* detection and age groups

			mcr1gene			
			positive	negative	Total	P-value
Age groups	10-30	Count	0	13	13	
		% of Total	.0%	26.0%	26.0%	
	31-50	Count	0	15	15	
		% of Total	.0%	30.0%	30.0%	
	51-70	Count	0	18	18	
		% of Total	.0%	36.0%	36.0%	0.008
	71-90	Count	1	3	4	
		% of Total	2.0%	6.0%	8.0%	
Total		Count	1	49	50	
		% of Total	2.0%	98.0%	100.0%	

4.4 Distribution of collected specimens

The isolates were collected from different sites; swabs (94%), blood (4%) and catheter tip (2%) as shown in (Table 3). Statically there was no association

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between the presence of *mcr-1* gene and the site that from which the bacteria was

Table (3): Relationship between *mcr-1* and sites of isolation

| | | | mcr1gene | | | |
			positive	negative	Total	P-value
Sample	swab	Count	1	46	47	
		% of Total	2.0%	92.0%	94.0%	
	blood	Count	0	2	2	
		% of Total	.0%	4.0%	4.0%	0.95
	catheter	Count	0	1	1	
		% of Total	.0%	2.0%	2.0%	
Total		Count	1	49	50	
		% of Total	2.0%	98.0%	100.0%	

isolated   (p-value = 0.95). Positive *mcr-1* gene was discovered among wound swabs specimen

4.5. Antimicrobial susceptibility testing

4.5.1 Susceptibility of Imipenem

There were 19 (38 %) isolates resistant to Imipenem and 31(62%) sensitive. There was no association between *mcr-1* gene and Imipenem (P-value = 0.42) as show in (Table 5).

Table (4): Relationship between *K.pneumoniae* resistant to

Imipenem and *MCR-1* gene detection

| | | | *mcr-1* gene | | | |
			positive	negative	Total	P-value
Imipenem	resistant	Count	0	19	19	
		% of Total	.0%	38.0%	38.0%	
	sensitive	Count	1	30	31	0.42
		% of Total	2.0%	60.0%	62.0%	
Total		Count	1	49	50	
		% of Total	2.0%	98.0%	100.0%	

4.5.2 Susceptibility of colistin

There were 20 (40%) isolates resistant to colistin and 30 (60%) were sensitive. There was no association between *mcr-1* gene and colistin (P-value = 0.40) as show in (Table 6).

Table (5): Relationship between *K.pneumoniae* resistant

to colistin and *MCR-1* gene detection

			mcr-1 gene		Total	P-value
			positive	negative	Total	P-value
Colisin	resistant	Count	0	20	20	
		% of Total	.0%	40.0%	40.0%	
	sensitive	Count	1	29	30	0.40
		% of Total	2.0%	58.0%	60.0%	
Total		Count	1	49	50	
		% of Total	2.0%	98.0%	100.0%	

4.5.3 Susceptibility of amikacin

There were 40(80%) isolates resistant to amikacin and 10 (20%) sensitive. There was significant association between *mcr-1* gene and amikacin (P-value = 0.043) as show in (Table 7).

Table (6): Relationship between *K.pneumoniae* resistant to amikacin and *MCR-1* gene detection

		mcr-1 gene		Total	

			positive	negative		P-value
Amikacin	Resistant	Count	0	40	40	
		% of Total	.0%	80.0%	80.0%	
	sensitive	Count	1	9	10	0.043
		% of Total	2.0%	18.0%	20.0%	
Total		Count	1	49	50	
		% of Total	2.0%	98.0%	100.0%	

4.5.4 Susceptibility of Cioprofloxacin

There were 41 (82%) isolates resistant to Cioprofloxacin and 9 (18%) sensitive. There was no association between *mcr-1* gene and Cioprofloxacin (P-value = 0.63) as show in (Table 8).

Table (7): Relationship between *K.pneumoniae* resistant to Cioprofloxacin and *MCR-1* gene detection

	mcr-1 gene	Total	

			positive	negative		P-value
Cioprofloxacin	resist	Count	1	40	41	
		% of Total	2.0%	80.0%	82.0%	
	sensitive	Count	0	9	9	
		% of Total	.0%	18.0%	18.0%	0.63
Total		Count	1	49	50	
		% of Total	2.0%	98.0%	100.0%	

4.5.5 Susceptibility of Ceftazidime

There were 45(90%) isolates resistant to Ceftazidime and 5(10%) sensitive. There was no association between *mcr-1* gene and Ceftazidime (P-value = 0.73) as show in (Table 9).

Table (8): Relationship between *K.pneumoniae* resistant to Ceftazidime and *MCR-1* gene detection

| | | | *mcr-1* gene | | | |
			positive	negative	Total	P-value
Ceftazidime	Resistant	Count	1	44	45	
		% of Total	2.0%	88.0%	90.0%	
	sensitive	Count	0	5	5	0.73
		% of Total	.0%	10.0%	10.0%	
Total		Count	1	49	50	
		% of Total	2.0%	98.0%	100.0%	

4.5.6 Susceptibility of amoxicillin

The all isolates organism (100%) resistant to amoxicillin and there were no sensitive isolates. There was no association between *mcr-1* gene and amoxicillin (P-value = 0.88) as show in (Table 10).

Table (9): Relationship between *K.pneumoniae* resistant to amoxicillin and the presence of *MCR-1* gene detection

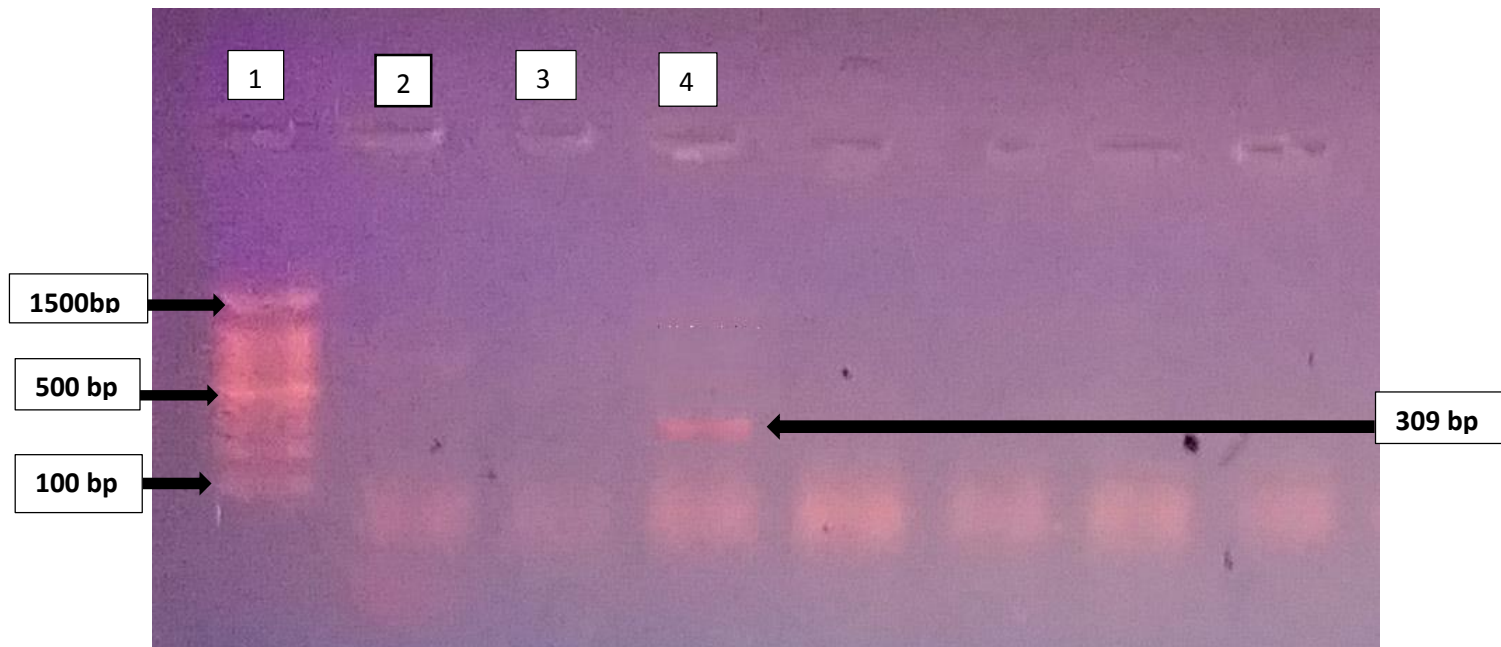|		mcr-1 gene			
		posiTive	negative	Total	P-value
Amoxicilin	resistant Count	1	49	50	
	% of Total	2.0%	98.0%	100.0%	
Total	Count	1	49	50	0.88
	% of Total	2.0%	98.0%	100.0%	

Figure 4.6: PCR amplification of *mcr-1*gene on 2% agarose gel electrophoresis.

Lane 1: DNA ladder: MW 100-1500bp.

Lane 2: Negative control

Lane 3: Negative result

Lane 4: showing typical bands size of 309 bp corresponding to the molecular size of *mcr-1* gene.

Chapter five

5.1 Discussion

Antibiotic resistance now is considered as one of major global crisis. In the last years the ability of rapid transmission of plasmid-mediated colistin resistant gene decrease the useful of colistin (polymyxins E) antibiotic as an important and last resort therapy against carbapenem-resistant and other multidrug resistant infection, in addition there were nearly to 22 genetic variation of *mcr-1* have been reported (El-Sayed *et al.*, 2020). However, the resistance of *Klebsiella pneumoniae* with moderate range (1.5–6.8%) and other *Enterobacterales* to polymyxins is increasing worldwide (Dalmolin *et al.*, 2018). Colistin resistance gene (*mcr*-1)was first described in *Enterobacteriaceae* isolated from animals, food and human in 2015 China, then it disseminated in Asia, Europe and North America, Egypt and Italy (Fernandes *et al.*, 2016).

In this study *mcr-1* was detedcted in a total of (50) *Klebsiella pneumoniae* isolates were collected from various hospitals at Khartoum State from June 2019 to September 2019, the results report the presence of mcr-1 gene in one sample (2%) from wound swab of old age female. In November 2017 Nehad Adam and Hisham N Altayb was published the first report of colistin resistance (mcr-1) gene in Khartoum-Sudan, they found gene in 7(14%) of isolates, mostly in *E.coli* (Nahed *et al.*, 2017).

Those findings indicate the spread of colistin resistance in Sudan manly in *Enterobacteriaceae,* mostly in *E.coli* with low occurrence in *Klebsiella pneumoniae.*

Most of the isolated organisms in this study were highly resistant to amikacin (90%), Ceftazidime (95%), ciprofloxacin (80%) and amoxicillin (100%). The study

showed no association between presence of *mcr-1* gene and age of the patients, gender and sample type (p- value >0.05).

The finding report the plasmid mediated colistin resistance (*mcr-1*) gene was detected in *K.pnemoniae* that sensitive to colistin by disc diffusion method, this result indicate "silent dissemination", the carriage of unexpressed genes is important and very rare phenomenon in antibiotic resistance. Resistant gene un expression mechanism is unknown yet but silencing may be result from different many ways include absence of effective promoter, low level of expression and change or mutation of the gene (Enne *et al.*, 2006). Other studies show that many of bacteria known as *mcr-1* positive are known to exhibit low level of resistance to colistin. The result expect that silent dissemination can increase risk of colistin resistant in which plasmid transfer is one of common mechanism of antibiotic resistance. In addition, it suggests that the unknown dissemination of antibiotic resistance gene in general and plasmid mediated colistin resistance gene may explain the presence of large number of untreatable infections specially that causes by multi drug resistant *enterobacteriaceae* in case of colistin resistance in Sudan. Although we expect the high resistance of above antibiotics may contribute in colistin resistance in some way.

In summary, this study reveals the "silent dissemination" of colistin resistance gene (*mcr-1*) in Khartoum in 2019 as first report to our knowledge of *K.pnemoniae* carries expression gene "silent gene". Our results expect that many of antibiotic resistance gene difficult to be detected and also cannot be detected by most laboratory tests as different types of sensitivity test and other phenotypic tests, Consequent of this, more wide dissemination of the resistant gene will occur, Additionally to unwariness with the real scale of problems which lead to minimize of suitable controlling.

5.2 Conclusion

The study conduct that the sensitivity of *K.pnemoniae* isolates varies in the tested different antibiotics.

The mcr-1 gene was detected in *K.pnemoniae* isolates in group of 70 years but with low frequency.

5.3 Recommendations:

1. Large sample size should be performed in other studies for more information of colistin resistance.

2. Other type of *mcr-1* and sub types *mcr-1* must be included in studies for detection of different type of plasmid mediated colistin resistance in Sudan.

3. Use of other technique for determination of *mcr-1* gene expression, assays of gene function and comparing phenotypic and genotypic gene resistance, such as microarray analysis, reverse transcription polymerase chain reaction (RT-PCR).

4. Gene sequencing and phylogenetic studies must be perform after detection of resistance genes for determination of novel mutation.

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Appendix 1

COLOURED PLATES

Color plate 1: Thermo-Cycler
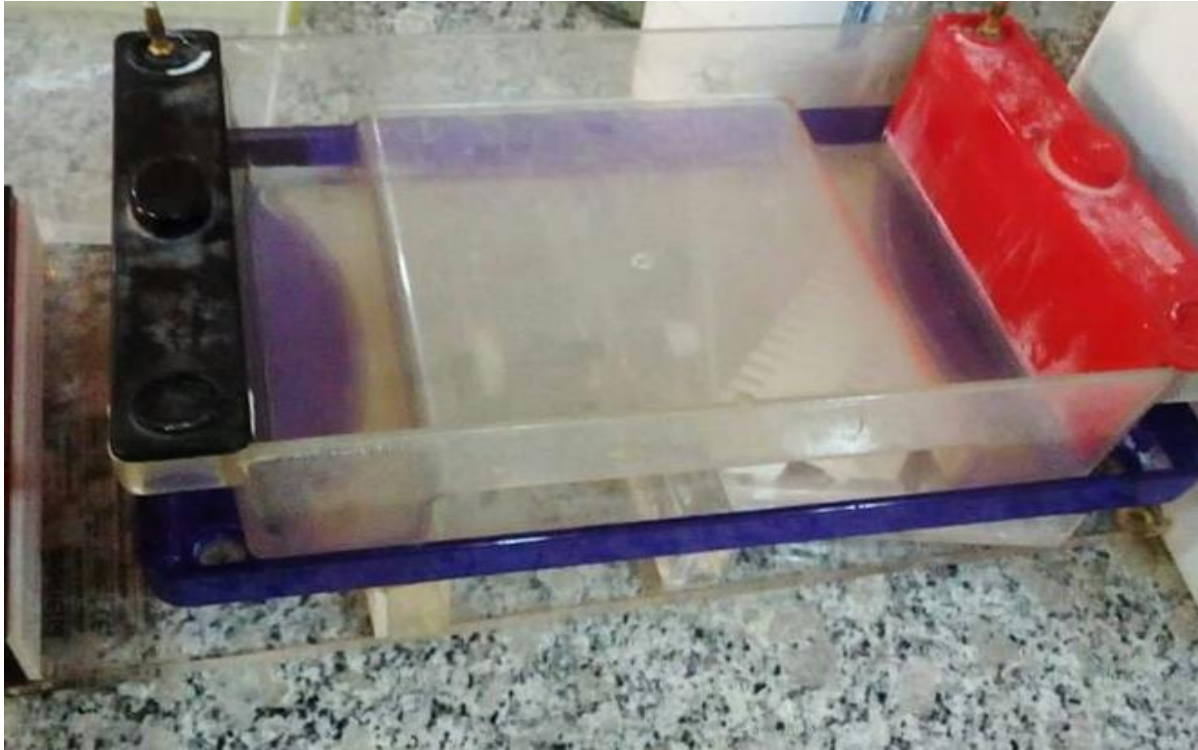
Color plate 2: Gel electrophoresis equipment's

Color plate 3: Microwave

Color plate 4: UVitec UK (Trans-illuminator)

Appendix 2

Preparation of reagents and culture media

1. Blood agar base (HIMEDIA, India)

Blood agar base is recommended as base to which blood may be added for use in theisolation and cultivation of fastidious pathogenic microorganisms.

Compositions

Ingredients Gms/L

Beef heart, infusion (beef extract)………………………..…… 5000

Tryptose …………………………………………………………..10

Sodium chloride ……………………………………………………….5

Final pH …………………………………………………............…7.3

Directions

Suspend 40 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min. Cool to 50°C and aseptically add 5% v/v sterile defibrinated blood. Mix well and pour into sterile

petridishes.

3. Kliger Iron Agar (KIA) (HIMEDIA, India)

KIA reactions are based on the fermentation of lactose and glucose (dextrose) and the production of hydrogen sulphide.

Compositions

Ingredients Gms/L

Peptic digest of animal tissue…………………………………….… 15

Yeast extract……………………………………….……………………3

Beef extract……………………………………………………….....3

Peptose peptone …………………………………….…….…….....5

Dextrose ……………………………………………....……………. 1

Lactose …………………………………………….….…………..10

Ferrous sulphate ……………………………………….… ……... 0.20

Sodium chloride ……………………………………….……..…..5

Sodium thiosulphate………………………………….….. .0.3

Phenol red…………………………………………....… …...0.042

Agar……………………………………………….……….. 15

Final pH (at 25°C)……………………………………...……7.4

Directions

Suspend 57.5 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min. mix and pour. Set as slope with butt.

4. Lugol,s iodine solution

To make one litter

Potassium iodine solution ……………………………………………20 g

Iodine ……………………………………………………….….. 10 g

Distilled water ……………………………………….……….to 1 litter

1. Weight the potassium iodine, and transfer to brown bottle pre marked to hold 1 litter.

2. Add about quarter of the volume of water, and mix until the potassium iodine solution is completely dissolved.

3. Weight the iodine, and add to potassium iodide solution. Mix until the iodine is dissolved.

4. Make up to 1 litter distilled water, mix well. Label the bottle and marked toxic.

Store at dark place

5. MacConkey Agar medium (HIMEDIA, India)

MacConkey Agar medium is a differential medium to distinguish between bacteria by neutral red indicator which changes colour when acid is produced following fermentation of lactose sugar.

Composition

Ingredients Gms/L

Peptic digest of animal tissue………………………………….…… 17

Protease peptone…………………………………………...…..……..… 3

Lactose……………………………………………………………... 10

Bile salts ……………………………………………………………1.5

Sodium chloride …………………………………………….…..…..5

Neutral red…………………………………………….………...…….03

Agar……………………………………………………….…….. 15

Final pH (at 25°C)…………………………………………...……………7.2

Directions

Suspend 51.53 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min. mix and pour.

6. McFarland Standard Turbidity tube 0.5:

Ingredients

Conc. Sulphuric acid ……………………….………………………1 ml

Dehydrated barium chloride ……………………..………………….0.5g

Distilled water ………………………..………………...……………99 ml

Prepare 1% V/V of sulphuric acid solution by adding 1 ml of concentrated sulphuric acid to 99 ml of DW and mix. Prepare 1% w/v solution of barium chloride by dissolve

0.5g of dehydrated barium chloride in 50 ml of distilled water. Add 0.6 ml of sulphuric acid then mix well.

Muller Hinton agar (HIMEDIA, India)

Muller Hinton agar is used for testing susceptibility of common and rabidly growing bacteria using antimicrobial disc, it manufactured to contain low level of thymine, thymidine, calcium and magnesium.

Compositions

Ingredients Gms/L

Casein acid hydrolysate ……………………………………………. 17

Beef heart infusion ……… …………………………………………… 2

Starch soluble …………………………………………………… 1.5

Agar…………………………………………………….…………17

Final pH (at 25°C)…………………………………………….…….... 7.3

Directions

Suspend 38 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min. mix and pour.

8. Nutrient agar (HIMEDIA, India)

Nutrient agar is used for cultivation of less fastidious organisms, can be enriched with blood or other biological fluids.

Compositions

Ingredients Gms/L

Peptone …………………….……………………..…………….…… 10

Beef extract ………. …………………………….…………….... 10

Sodium chloride ………………………………………………………5

Yeast xtract……………………………………………….…………1.5

Agar…………………………………………………….…………15

Final pH (at 25°C)………………………………………….... 7.3

Directions

Suspend 28 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min. mix and pour.

10. Peptone water

Used for culturing organisms to proceed indole test in the presence of Kovac's or Ehrlich's reagent that reacts with the indole to produce a red coloured compound.

Compositions

Ingredients Gms/L

Peptic digest of animal tissue…………………………..………… 10

Sodium chloride …………………………………………………..5

Final pH (at 25°C)………………………………………………7.2

Directions

Suspend 15 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min. mix and pour.

11. Simmons citrate Agar (HIMEDIA, India)

This test is used to assist in the identification of enterobacteria. The test is based on the ability of an organism to use citrate as its only source of carbon.

Compositions

Ingredients Gms/L

Magnesium sulphate …………………………………………… 0.20

Ammonium dihydrogenphosphate …………………...……………… 1

Dipotassium phosphate ……………………………...…………... 1

Sodium citrate ……………………………………………………..2

Sodium chloride …………………………………..………….5

Bromothymol blue ………………………………...………….0.08

Agar………………………………………………………….. 15

Final pH (at 25°C)…………………………………..…...………6.8

Directions

Suspend 24.28 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min. mix and pour. Set as slope.

12. Urea Agar Base (Christensen) (HIMEDIA, India)

Testing for Urease enzyme activity is important in differentiating enterobacteria. Especially for *proteusspp*

Compositions

Ingredients Gms/L

Peptic digest of animal tissue…………………………...…..……… 1

Dextrose ……………………………………………………………. 1

Disodium phosphate ………………………………………... 1.20

Monopotassiumphosphate ……………………………….…….0.80

Sodium chloride …………………………………………………….5

Phenol red……………………………………………..……..… 0.012

Agar…………………………………………………………….15

Final pH (at 25°C)…………………………………………….....6.8

Directions

Suspend 24 grams in 950 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 10 ibs pressure (115°C) for 20 min. Cool to 50°C and a aseptically add 50 ml of sterile 40% of urea solution (FD048) and mix.