Sudan University of Science and Technology

College of Graduate Studies

Antimicrobial Susceptibility and Molecular Detection of Carbapenem Resistance Genes of *Proteus mirabilis* Isolated from Clinical Specimens in Khartoum State-Sudan

اختبار حساسية المضادات الحيوية والكشف الجزيئي لجينات مقاومة الكاربابينيم للمتقلبة الرائعة المعزولة من عينات سريرية في ولاية الخرطوم - السودان

A dissertation submitted in partial fulfillment for the requirements of M.Sc. Medical Laboratory Science (Microbiology)

Submitted By:

Loma Awad Hamadalneel Almubarak

B.Sc. (Honors) Medical Laboratory Science (Microbiology), Sudan University of Science and Technology- (2016)

Supervisor:

Dr. Hind Haidar Ahmed

December, 2020

الآية

قَالَ تَعَالَىٰ:

﴿يَٰبُنَيَّ إِنَّهَآ إِن تَكُ مِثۡقَالَ حَبَّةٖ مِّنۡ خَرۡدَلٖ فَتَكُن فِي صَخۡرَةٍ أَوۡ فِي ٱلسَّمَٰوَٰتِ أَوۡ فِي ٱلۡأَرۡضِ يَأۡتِ بِهَا ٱللَّهُۚ إِنَّ ٱللَّهَ لَطِيفٌ خَبِيرٞ ١٦﴾

(سورة لقمان: الاية ١٦)

DEDICATION

I dedicate this work to…

My LovelY Parents, my family,

my friends and my colleagues….

ACKNOWLEDGEMENTS

Firstly, thanks to **ALMIGHTY ALLAH** for giving me patience and strength to complete this work.

Special thanks to my respectable supervisor **Dr. Hind Haider Ahmed**, for her encouragement, follow up and guidance through the research.

I would like to express my gratitude to **Dr. Hisham Noraldayem Altayeb, Dr. Abdelrahman Ali, Dr. Samar Mohammed Saeed, Ustaz. Asgad Mohammed, Duaa Tarig.**

My thanks also extended to Medical Microbiology Laboratory staff members. Finally great thanks to my colleagues and friends for their fruitful comments.

ABSTRACT

Carbapenems are the most powerful β-lactam antibiotics against bacteria especially multidrug resistant isolates. Since there were no comprehensive studies about prevalence of carbapenem resistance genes in Sudan, so this study was conducted to detect carbapenem resistance genes of *Proteus mirabilis* from clinical specimens in Khartoum State during the period from March to November 2019.

A total of fifty (n=50) *Proteus mirabilis* isolates were included in this descriptive case laboratory-based study. *P.mirabilis* were isolated from urine, wound swab, high vaginal swab and semen samples which were obtained from different hospitals in Khartoum State. The isolated organisms were stored in 20% Glycerol peptone media and were inoculated on nutrient agar and MacConkey's agar, and reidentified by using biochemical tests. Antibiotic susceptibility tests were carried out using Kirby Bauer disc diffusion technique. DNA was extracted using GenoLyse method, then were tested for the presence of carbapenem resistance genes (NDM, IMP, VIM, OXA-48 and KPC) using Multiplex PCR.

Out of fifty isolates, 48(96%) were resistant to ceftazidime, 31(62%) were resistant to ciprofloxacin, 30(60%) were resistant to amikacin, 43(86%) were resistant to gentamicin and 13(26%) were resistant to imipenem.

All 50 *P.mirabilis* isolates investigated were negative 0 (0%) for all five carbapenem resistance genes.

This study concluded that, there is no carbapenem resistance genes (NDM, IMP, VIM, OXA-48 and KPC) among *P.mirabilis* isolates in Khartoum State.

الخلاصة

الكاربابينيمات (بيتا لاكتام) منأقوى المضادات الحيوية ضد البكتيريا وخاصة المقاومة للأدوية. ونظرا لعدم وجود دراسات شاملة حول انتشار الجينات المقاومة للكاربابينيم في السودان لذا أجريت هذه الدراسة للكشف عن جينات مقاومة الكاربايبنيم للمتقلبة الرائعةالمعزولة من عينات سريرية في ولاية الخرطوم في الفترة من مارس الى نوفمبر 2019.

شملت هذه الدراسة الوصفية خمسين (ن= 50) من المتقلبة الرائعة المعزولة من عينات البول، الجروح، المسحة المهبلية العالية والمني التي جمعت من مختلف المستشفيات في ولاية الخرطوم. تم حفظ السلالات المعزولة في وسط الجليسرول بيبتونبتركيز 20 % لحين زرعها على الاجار المناسب لها وتم التعرف عليها باستخدام التفاعلات الكيموحيوية. أجريت اختبارات الحساسية للمضادات الحيوية باستخدام تقنية انتشار القرص(كيربي باور). استخلص الحمض الوراثي النووي باستخدام طريقة الجينولايس ومن ثماختبارها لوجود جينات مقاومة الكاربابينيم (NDM, IMP, VIM, OXA-48 and KPC) باستخدام تفاعل البلمرة التسلسلي المتعدد.

من بين خمسين متقلبة رائعة معزولة وجد ان 48(96%) كانت مقاومة للسيفتازيديم ،31(62%) كانت مقاومة للسيبروفلوكساسين،30(60%) كانت مقاومة للاميكاسين ،43(86%) كانت مقاومة للجنتامايسين و13(26%) كانت مقاومة للايميبينيم.

كل المتقلبة الرائعة المعزولة التي فحصت لوجود جينات مقاومة الكاربابينيم الخمسة كانت سلبية 0(0%).

خلصت هذه الدراسة الى انه لا يوجد جينات مقاومة الكاربايبنيم (NDM, IMP, SPM, OXA-48 and KPC) في المتقلبة الرائعة بولاية الخرطوم.

LIST OF CONTENTS

Subjects		Page No.
الآية		I
Dedication		II
Acknowledgements		III
Abstract, English		IV
Abstract, Arabic		V
List of contents		VI
List of tables		X
List of figures		XI
List of appendices		XII
List of abbreviations		XIII
CHAPTER ONE: INTRODUCTION		
1.1	Introduction	1
1.2	Rationale	3
1.3	Objectives	4
1.3.1	General objective	4
1.3.2	Specific objectives	4
CHAPTER TWO: LITERATURE REVIEW		
2.1	*Proteus mirabilis* (*P.mirabilis*)	6
2.1.1	General characteristics	6
2.1.2	Culture and metabolic characteristics	6
2.1.3	Clinical significance	7

2.1.4	Virulence factors	7
2.2	Carbapenems	7
2.2.1	Mechanisms of resistance against carbapenems	9
2.3	Carbapenemases	9
2.3.1	Classification	9
2.3.1.1	Functional classification	9
2.3.1.2	Molecular classification	10
2.3.1.2.1	Class A Serine Carbapenemases	10
2.3.1.2.2	The *Klebsiella pneumoniae* carbapenemases enzyme (KPCs)	11
2.3.1.2.3	Molecular Class B enzymes metallo-Beta-lactamases (MBLs)	11
2.3.1.2.4	The Active on Imipenem enzyme (IMP)	12
2.3.1.2.5	The Verona Integron encoded Metallo-β-lactamase enzymes (VIM)	13
2.3.1.2.6	The New Delhi Metallo-β-lactamase enzymes (NDM)	13
2.3.1.2.7	Molecular Class D enzymes	14
2.3.2	Laboratory detection of carbapenemases	14
2.3.2.1	Screening tests	14
2.3.2.1.1	Screen agars	15
2.3.2.2	Phenotypic detection of carbapenemase activity	15
2.3.2.2.1	Modified Hodge test (MHT)	15
2.3.2.2.2	Synergy test	16

2.3.2.2.3	Carba NP test	16
2.3.2.3	Molecular detection of carbapenemases genes	17
2.3.2.4	Previous studies	18
CHAPTER THREE: MATERIALS AND METHODS		
3.1	Study design	21
3.2	Study area	21
3.3	Study duration	21
3.4	Study sample	21
3.5	Ethical considerations	21
3.6	Sampling technique	21
3.6.1	Sample size	21
3.7	Data collection	21
3.8	Laboratory methods	22
3.8.1	Samples preservation	22
3.8.2	Subculture	22
3.8.3	Reidentification of isolates	22
3.8.3.1	Gram stain	22
3.8.3.2	Biochemical tests	22
3.8.3.2.1	Indole test	22
3.8.3.2.2	Kligler Iron Agar (KIA)	23
3.8.3.2.3	Urease test	23
3.8.3.2.4	Citrate utilization test	23
3.8.4	Susceptibility testing	23
3.8.5	Detection of carbapenem resistance genes of *P.mirabilis*	24
3.8.5.1	DNA extraction	24
3.8.5.2	PCR technique	24

3.8.5.2.1	Primers	24
3.8.5.2.2	Preparation of 10X Tris Base EDTA (TBE) buffer	25
3.8.5.2.3	Preparation of 1X TBE buffer	25
3.8.5.2.4	Preparation of ethidium bromide	25
3.8.5.2.5	Preparation of agarose gel	25
3.8.5.2.6	Master Mix	26
3.8.5.2.7	Preparation of reaction mixtures	26
3.8.5.2.7.1	Reaction mixture for carbapenem resistance genes	26
3.8.5.2.8	Amplification Protocol of NDM, IMP and VIM	26
3.8.5.2.8.1	Amplification Protocol of KPC and OXA-48 genes	26
3.8.5.2.9	Visualization of PCR product	26
3.9	Data analysis and presentation	26
CHAPTER FOUR: RESULTS		
4.1	Distribution of specimen types	28
4.2	Results of Antibiotics susceptibility test	28
4.3	Detection of *P.mirabilis* carbapenem resistance genes	30
CHAPTER FIVE :DISCUSSION, CONCLUSION, AND RECOMMENDATIONS		
5.1.	Discussion	32
5.2	Conclusion	34
5.3	Recommendations	35
	References	36
	Appendices	46

LIST OF TABLES

NO.	Titles	Page No.
3.1	Primers sequences and amplicons size used for detection of carbapenem resistance genes of *P.mirabilis*	25
4.1	Results of Antibiotics susceptibility test	29

LIST OF FIGURES

No	Title	Page No.
4.1	Distribution of *P.mirabilis* according to the type of specimens	28
4.2	Gel electrophoresis of carbapenem resistance genes (NDM, IMP, VIM, OXA-48 and KPC)	30

LIST OF APPENDICES

NO.	Title	Page No.
1	Colored plate 1:Biochamical reaction of *P.mirabilis*	51
2	Colored plate 2: Susceptibility test results	52
3	Colored plate 3: Microwave	53
	Colored plate 4: Thermocycler	53
4	Colored plate 5: Gel electrophoresis and power supply device	54
5	Colored plate 6: Transilluminator system	54
6	Colored plate 7:Microcenterfuge device	55

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CAZ	Ceftazidime
CLSI	Clinical and Laboratory Standards Institute
CPE	Carbapenemase-producing Enterobacteriaceae
CRE	Carbapenem Resistant *Enterobacteriaceae*
EDTA	Ethylene diamaine tetraacetic acid
ESBLs	Extended spectrum beta-lactamases
GES	Guiana extended spectrum
IMI	Imipenem-hydrolyzing β-lactamase
IMP	Imipenemase
KIA	Kligler Iron Agar
KPC	*Klebsiella pneumoniae* Carbapenemase
MBLs	Metallo-beta-lactamases
MDR	Multidrug resistant
MHT	Modified Hodge test
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *S. aureus*
NDM	New Delhi Metallo-betalactamase
NMC-A	Not metalloenzyme carbapenemase
NS	Normal Saline
OMPs	Outer membrane proteins
OXA-48	Oxacillinase-48
PBPs	Penicillin binding proteins
PCR	Polymerase Chain Reaction
SME	*Serratia marcescens* enzyme
SPSS	Statistical Packagefor Social Science

TBE	Tris Base EDTA
UTI	Urinary tract infections
VFs	Virulence factors
VIM	Verona Integron Metallo-betalactamases

CHAPTER ONE

INTRODUCTION

CHAPTER ONE
INTRODUCTION

1.1. Introduction

The genus *Proteus* belongs to the family Enterobacteriaceae (Tsai *et al*., 2014). It is motile, Gram-negative rods with peritrichous flagella **(**Manos and Belas**,** 2006), is well-known for its urease production and distinctive ability to differentiate into elongated swarm cells and characteristic bull's-eye pattern of motility on agar plates (Armbruster *et al*., 2018).

P.mirabilis is an opportunistic pathogen that can cause diarrhea, septicemia, meningitis, urinary tract and respiratory system infections (Hu *et al*., 2012).

For a long time, *Proteus* was known to be susceptible to beta-lactam antibiotics (Musa *et al*., 2019). Nowadays they are becoming resistant due to acquire genes encoding multiple antibiotic resistance mechanisms, including extended-spectrum Beta -lactamases (ESBLs), AmpCs, and carbapenemases (Lutgring and Limbag, 2016).

Increasing prevalence of antibiotic resistance and the lack of new antibiotic drug development has gradually reduced the treatment options for bacterial infections (Lee *et al*., 2016).

Carbapenems often are used as the most appropriate agents in the treatment of infections caused by multi resistant Gram-negative bacteria, the most common carbapenemases to emerge in recent years have been the *Klebsiella pneumoniae* carbapenemases (KPCs), these plasmid-carried Ambler class A enzymes have since been identified in multiple genera and species of the Enterobacteriaceae **(**Shen *et al.*, 2015*).* Carbapenemase-producing Enterobacteriaceae (CRE) have spread worldwide and caused a significant public health alert (Chen *et al*., 2015). Carbapenems such as imipenem and meropenem are recommended as first-line therapy for

severe infections caused by Enterobacteriaceae producing extended spectrum β lactamases (ESBLs) (Nordmann *et al*., 2009). Carbapenems have the broadest spectra among all β-lactams and are primarily used to treat infections by multi-resistant Gram-negative bacteria, the emergence and spread of carbapenemases became a major public health concern (Jeon *et al*., 2015).

Carbapenemases are the most versatile family of β-lactamases that are able to hydrolyze carbapenems and many other β-lactams (Jeon *et al*., 2015). According to the dependency of divalent cations for enzyme activation, carbapenemases can be divided into metallo-carbapenemases (zinc-dependent class B) and non-metallo-carbapenemases (zinc-independent classes A, C, and D) β-Lactamases are bacterial enzymes that hydrolytically inactivate β-lactam antibiotics and are a major cause of the emergence of pathogenic bacteria resistant to β-lactam antibiotics such as penicillins, cephalosporins, monobactams, and carbapenems (Jeon *et al*., 2015).

Carbapenemase genes is of particular concern within bacterial species exhibiting intrinsic antibiotic resistance, such as *P. mirabilis* (Chen *et al*., 2015). The spread of carbapenemase-producing Enterobacteriaceae (CPE) over the last decades is a great danger because carbapenems are the last treatment options for infections caused by MDR bacteria (Fursova *et al.*, 2015).

While decreased susceptibility to imipenem is intrinsic in Proteeae, some *P.mirabilis* exhibit an increased level of resistance to imipenem (with MICs ranging from 16 to 64 mg/L) due to loss of outer membrane (Porin *et al*., 2014). The acquisition of blaKPC by *P. mirabilis* represents a rare event, and only a few descriptions have been reported to date. (Di Pilato *et al.*, 2016).

1.2. Rationale

Antibiotic resistance is now a linked global problem, it increases the morbidity, mortality and costs of treating infectious diseases (Hawkey and Jones, 2009). Carbapenem resistance, mainly among Gram-negative pathogens spreading rapidly causing serious outbreaks and limiting treatment options (Meletis, 2016). *P.mirabilis* is a common cause of community and healthcare-associated infections, Patients with infections caused by such pathogen is associated with delay in appropriate antimicrobial therapy due to multiple drug resistance (Tsai *et al*., 2014). The dissemination of resistance is associated with genetic mobile elements, such as plasmids, that may also carry virulence determinant (da Silva and Mendonça, 2012). Considering the fact that there were no comprehensive studies about prevalence of carbapenem resistance genes in Sudan so detection of those genes in *P.mirabilis* from clinical specimens will be of a great value. Such data serve an important role in understanding the spread of this pathogen.

1.3. Objectives

1.3.1. General objective

To study carbapenem resistant genes of *Proteus mirabilis* from different clinical specimens in Khartoum State.

1.3.2. Specific objectives

1- To reidentify *P.mirabilis* isolates using convencial techniques.

2- To determine the susceptibility of *P.mirabilis* to antimicrobial agents using disc diffusion method.

3- To detect the carbapenem resistant genes of *P.mirabilis* (KPC, OXA48, NDM, IMP, and VIM) using multiplex PCR.

CHAPTER TWO

LITREATURE REVIEW

CHAPTER TWO
LITERATURE REVIEW

2.1. *Proteus mirabilis* (*P.mirabilis*)

2.1.1. General characteristics

The genus *Proteus* along with genus *Providencia* and *Morganella* belongs to the tribe Proteeae of the family Enterobacteriaceae (Pal *et al*., 2014), it consists of four named species (*P.mirabilis*, *P.penneri*, *P.vulgaris*, *P.myxofaciens*) (Pal *et al*., 2014). *P.mirabilis* is a Gram-negative bacterium which is well-known for its ability to swarm across surfaces in a striking bulls'-eye pattern (Al-Jumaily and Zgaer, 2016), it is motile Gram-negative enteric bacterium (Wang *et al*., 2006). *Proteus spp* are the causative agent of a variety of opportunistic nosocomial infections including those of the respiratory tract, ear, nose, skin, burns, and wounds, it may also cause gastroenteritis (Jacobsen *et al*., 2008). It commonly associated with urinary tract infections (UTIs) in those individuals with structural or functional abnormalities, especially ascending infections in patients undergoing urinary catheterization (Jacobsen *et al*., 2011).

2.1.2. Culture and metabolic characteristics

The *proteus* isolates were firstly identified as related to the genus *Proteus* by swarming phenomenon on blood agar and the bacteria on the MacConkey agar appeared pale (Al-Kazaz and Al-Bassam, 2013). The colones are single pale colonies on MacConkey agar, are medium in size and the edges smooth and non ferment sugar lactose as well as the smell of bacterial growth which is similar to smell of fish (Ali and Yousif, 2015). *Proteus mirabilis* isolates are oxidase negative, indol negative, catalase positive, urease positive and variable citrate utilization (Ali and Yousif, 2015).

2.1.3. Clinical significance

Proteus rods are opportunistic bacterial pathogens which under favorable conditions cause urinary tract infections (UTIs), wound infections, meningitis in neonates or infants and rheumatoid arthritis (Różalski *et al*., 2012). These bacteria are the causative agents of a variety of opportunistic nosocomial infections including those of the respiratory tract, eye, ear, nose, skin, burns, throat and wounds (Jacobsen *et al*., 2011).

2.1.4. Virulence factors

Virulence factors (VFs) comprise mechanisms allowing pathogenic bacteria to cause infections (Gharrah *et al.*, 2017).The severity of any infection caused by the members of the genus *Proteus* depends mainly on the availability of virulence factors that may include β-Lactamase, extended spectrum β-lactamases, protease, urease and hemolysin production, swarming motility, adhesion and biofilm formation (Hasan *et al*., 2011). All these factors collectively or separately play important roles in pathogenicity and pathogenesis of disease (Wang *et al*., 2006). The ability of *P.mirabilis* to express virulence factors, including urease and haemolysin, to invade human urothelial cells, is coordinately regulated with swarming differentiation (Liaw *et al*., 2004).

2.2. Carbapenems

The term "carbapenem" is defined as the 4:5 fused ring lactam of penicillins with a double bond between C-2 and C-3 but with the substitution of carbon for sulfur at C-1 (Papp-Wallace *et al*., 2011). Carbapenems belong to the β-lactam family of antibiotics, which is the biggest and most important class of clinical antibiotics (Coulthurst *et al*., 2005). β-Lactams inhibit bacterial cell wall peptidoglycan biosynthesis by the formation of crosslinks, a process that is catalysed by transpeptidase enzymes (Coulthurst *et al*., 2005). β-Lactams are thought to function as structural analogues of the transpeptidase substrate, forming relatively

stable complexes with the enzyme ,this, in turn, inhibits crosslink formation by the enzyme, and the resulting loss in cell wall integrity can lead to lysis of growing bacterial cells (Coulthurst *et al*., 2005). Carbapenems used to treat serious infections in hospital settings (Bedenić *et al*., 2014), it possesses the broadest spectrum of activity against Gram-positive and Gram-negative bacteria (Papp-Wallace *et al*., 2011). Carbapenems are β-lactam antibiotics characterised by the presence of a β-lactam ring with a carbon instead of sulfone in the 4-position of the thyazolidinic moiety (Nicoletti *et al*., 2002). The first carbapenem to be utilised in therapy was imipenem, the N-formimidoyl derivative of thienamycin, it coadministered with cilastatin, an inhibitor of human renal dehydropeptidase I, as imipenem is hydrolysed by this enzyme (Nicoletti *et al*., 2002). Meropenem was the first carbapenem with a 1-β-methyl group and 2-thio pyrrolidinyl moiety, which renders this antibiotic stable to renal dehydropeptidase I (Nicoletti *et al*., 2002). Other carbapenems for parenteral administration later discovered include biapenem, panipenem, ertapenem, lenapenem, E-1010, S-4661 and BMS-181139. Carbapenems which are orally administered include sanfetrinem, DZ-2640, CS-834 and GV-129606 (Nicoletti *et al*., 2002). Similar to penicillin and cephalosporins, the carbapenems are bactericidal agents that bind to the penicillin binding proteins (PBPs) inhibiting the bacterial cell wall synthesis, they show less resistance than other beta-lactams because of their stability to hydrolysis by many extended-spectrum chromosomal and plasmid-mediated beta-lactamases, including AmpC and extended-spectrum beta-lactamases (ESBLs) (Zhanel *et al*., 2007). Despite the broad-spectrum, the carbapenems globally lack activity against *Enterococcus faecium*, methicillin resistant *Staphylococcus aureus* (MRSA) and *Stenotrophomonas maltophilia*. Among them, ertapenem is

also limited by the lacking activity against *Pseudomonas aeruginosa, Acinetobacter spp.* and *Enterococcus* spp (Bassetti *et al*., 2009).

2.2.1. Mechanisms of resistance against carbapenems

The main mechanisms of resistance to carbapenems in CRE include hydrolysis of carbapenems by a plasmid-encoded carbapenemase, impaired outer membrane permeability due to inactivation of particular porins (i.e., OmpC and OmpF in *E. coli* and their analogs) coupled with high-level expression of cephalosporinases such as AmpC and/or extended spectrum β-lactamase (ESBL), or a combination of these mechanisms (Senchyna *et al*., 2018). Resistance to beta-lactam antibiotics is mediated through enhanced degradation by beta-lactamases, alteration in penicillin-binding proteins, changes in outer membrane porins for decreased permeability, and expulsion of antibiotics out of cell through efflux pump (Asif and Rehman, 2018).

2.3. Carbapenemases

Carbapenemases are specific beta-lactamases with the ability to hydrolyze carbapenems and cause carbapenem resistance (Papp-Wallace *et al*., 2011).

2.3.1. Classification

Classification of β-lactamases can be defined according to two properties, functional and molecular (Queenan and Bush, 2007).

2.3.1.1. Functional classification

The functional classification proposes to classify the known beta-lactamases into four major functional groups (Groups 1-4) according to the group specific substrate or inhibitor profiles, group 2 is differentiated into multiple subgroups (Dahiya *et al*., 2015).The updated system comprises Group 1 (Class C) cephalosporinases; Group 2 (Classes A and D) broad-spectrum, inhibitor resistant, and ESBLs and serine carbapenemases; and Group 3 metallo-beta-lactamases (MBLs). In this functional classification

scheme, carbapenemases are found primarily in Groups 2f and 3 (Queenan and Bush, 2007).

2.3.1.2. Molecular classification

Ambler is based on molecular structure. In this classification, carbapenemases may belong to classes A, B, or D. Class A and D. are serine carbapenemases, meaning that they have a serine at their active sites, like extended spectrum beta lactamases (ESBLs). In contrast, the class B enzymes are known as metallo-b-lactamases, because they require zinc as a cofactor (Munoz-Price and Quinn, 2009).

Group B enzymes require one or two Zn cations for activity and are subdivided into three groups, B1, B2, and B3, based on sequence alignments and structural analysis, subclasses B1 and B3 have two zinc ions whereas subclass B2 has only one zinc ion. B2 enzymes are preferentially carbapenemases whereas B1 and B3 enzymes have broad hydrolysis spectrum (Papp-Wallace *et al*., 2011).

2.3.1.2.1. Class A Serine Carbapenemases

Class A serine carbapenemases are members of functional group 2f, they can hydrolyze a broad variety of beta-lactams,including carbapenems, cephalosporins, penicillins, and aztreonam. However, they all are inhibited by clavulanate and tazobactam (Dahiya *et al*., 2015). The enzymes characterized from *Enterobacteriaceae* include not metalloenzyme carbapenemase-A (NMC-A), *Serratia marcescens* enzyme (SME) 1-3, "imipenem-hydrolysing beta-lactamase" (IMI-1), *K. pneumoniae* carbapenemases 1-3 (KPC), and Guiana extended-spectrum (GES-2) (Dahiya *et al*., 2015).SME was first detected in England from two *S. marcescens* isolates that were collected in 1982, IMI and NMC-A enzymes have been detected in rare clinical isolates of *E. cloacae* in the United States, France, and Argentina (Queenan and Bush, 2007). GES (for Guiana extended spectrum): this enzyme was first observed in a

K.pneumoniae isolate from French Guiana in 1998 (Rasmussen and Høiby, 2007). The enzymes of the GES family differ from each other by 1-4 amino acid substitutions (Papp-Wallace *et al*., 2011). The genes encoding the class A carbapenemases are either plasmid-borne or located on the chromosome of the host (Rasmussen and Høiby, 2007). The genes for SME, IMI, and NMC-A beta-lactamases are chromosomally located while the KPC and GES family of carbapenemases are plasmid encoded (Dahiya *et al*., 2015). Characteristic properties of these enzymes include presence of an active-site serine at position 70 and presence of a disulfide bond between Cys69 and Cys238 (changes the overall shape of the active site) (Papp-Wallace *et al*., 2011).

2.3.1.2.2. The *Klebsiella pneumoniae* carbapenemases enzyme (KPCs)

Klebsiella pneumoniae carbapenemases (KPCs) were first identified in 1996 in a *K.pneumoniae* isolate obtained from a patient hospitalized in North Carolina, USA (Findlay *et al*., 2016). The KPC carbapenemases differ from the other functional group 2f enzymes by two important characteristics, first is the presence of the KPC enzymes on transferable plasmids,the second, their substrate hydrolysis spectrum includes the aminothiazole oxime cephalosporins, such as cefotaxime (Queenan and Bush, 2007). Although, the KPC beta-lactamases are predominantly found in *K. pneumoniae*, there have been reports of these enzymes in *Enterobacter* species and in *Salmonella* spp (Dahiya *et al*., 2015).

2.3.1.2.3. Molecular Class B enzymes metallo-Beta-lactamases(MBLs)

MBLs are a group of class B beta-lactamases possessing a metal ion required for beta-lactam hydrolysis (Patel and Bonomo, 2011), this class of beta-lactamases can hydrolyze carbapenems and known by its resistance to the commercially available beta-lactamase inhibitors, but susceptibility to inhibition by metal ion chelators (Dahiya *et al*., 2015), in addition to the carbapenems, most of these enzymes can hydrolyze cephalosporins and

penicillins but lack the ability to hydrolyze aztreonam (Queenan and Bush, 2007). The mechanism of hydrolysis is dependent on interaction of the beta-lactams with zinc ions in the active site of the enzyme, resulting in the distinctive trait of their inhibition by EDTA, a chelator of Zn2 and other divalent cations (Dahiya *et al*., 2015). Class B enzymes are generally chromosomally encoded isolated from *Stenotrophomona smaltophilia*, *Aeromonas spp*., *Bacillus cereus*, *Bacteroides fragilis*, *Flavobacterium spp* and *Legionella gormanii*) (Bassetti *et al*., 2009). The more geographically widespread MBLs include IMP, VIM and, most recently, NDM-1 (Patel and Bonomo, 2011). The metallo-beta-lactamases belong to the IMP, VIM families have been detected primarily in *P.aeruginosa*; however, there are increasing numbers of reports worldwide of this group of beta-lactamases in the Enterobacteriaceae (Bassetti *et al*., 2009).

2.3.1.2.4. The Active Imipenem enzyme (IMP)

These MBLs are located within a most of genetic structures, most commonly integrons, which can be incorporated into gene cassettes (Patel and Bonomo, 2011) Gene cassettes are small pieces of circular DNA, approximately 1 kb in size, comprising a single gene together with a recombination site termed a 59-base element (Walsh *et al*., 2005). In 1988, IMP-1, a plasmid-mediated MBL, was identified in an isolate of *P. aeruginosa* from Japan (Patel and Bonomo , 2011), this enzyme hydrolyzed imipenem, penicillins, and extended spectrum cephalosporins but not aztreonam (Dahiya *et al*., 2015).At the present time, 26 variants of IMP are described with most cases of IMP-mediated carbapenem resistance being reported from Asia an among *P. aeruginosa* (Patel and Bonomo, 2011).

2.3.1.2.5. The Verona Integron encoded Metallo-β-lactamaseenzymes (VIM)

VIM-1 was first isolated in Verona, Italy, in 1997, VIM-2 was identified in France in 1996 (Dahiya *et al*., 2015). To date, 23 variants of VIM are found and VIM-2 appears to be the most common MBL isolated worldwide (Patel and Bonomo, 2011).These enzymes were primarily detected in *P.aeruginosa*; however, there were increasing numbers of reports worldwide of this group of beta-lactamases in the Enterobacteriaceae (Bassetti *et al*., 2009). These enzymes are resistant to a series of β- lactams, including piperacillin, ceftazidime, imipenem, and aztreonam (Walsh *et al*., 2005).

2.3.1.2.6. The New Delhi Metallo-β-lactamaseenzymes (NDM)

New Delhi metallo beta-lactamase (NDM) is a type of Metallo beta_lactamase (MBL) able to hydrolyze most Beta-lactams (including carbapenems) but not monobactams (Wu *et al*., 2019), It was first identified in a *Klebsiella pneumoniae* strain isolated from a Swedish patient who had been hospitalized in New Delhi, India, in 2008 (Dortet *et al*., 2014a), NDM-1 has been found in various species of the *Enterobacteriaceae*, *Acinetobacter*, and *Pseudomonas*, and 24 variants of NDM have been identified (Wu *et al*., 2019). NDM-positive strains are usually resistant to most antimicrobial agents in addition to Beta-lactams due to the co-existence of other resistance mechanisms (Nordmann *et al*., 2011). NDM-positive strains cause a variety of infections that have been reported to be associated with high mortality rates (Guducuoglu *et al*., 2018). NDM-positive strains have been found worldwide, representing a significant challenge for clinical management and public health (Moellering, 2010).

2.3.1.2.7. Molecular Class D enzymes

Class D beta-lactamases, also known as oxacillinases or OXA type beta-lactamases (OXAs), are active-serine-site enzymes like Ambler class A and class C beta-lactamases, differing from class A and C enzymes in amino acid structure, whereas class B beta-lactamases are metalloenzymes with a Zn2 ion in the active site (Poirel *et al*., 2010). These beta-lactamases can hydrolyze cloxacillin or oxacillin at a rate of>50% than for benzylpenicillin and hence are known as OXA enzymes (Dahiya *et al*., 2015). Class D beta-lactamases are usually not inhibited by clavulanic acid, tazobactam, and sulbactam, whereas their activities may be inhibited in vitro by sodium chloride (NaCl) (Poirel *et al*., 2010). OXA related enzymes now comprise the second largest family of beta-lactamases (Dahiya *et al*., 2015).

2.3.2. Laboratory detection of carbapenemases

Detection of carbapenemase-producing organisms in the clinical microbiology laboratory is a major importance for the choice of appropriate therapeutic schemes and the implementation of infection control measures (Miriagou *et al.*, 2010). The detection strategy includes a screening step followed by a phenotypic and genotypic confirmation step (Stuart and Leverstein-Van Hall, 2010).

2.3.2.1. Screening tests

Screening is based on detection of reduced susceptibility to carbapenems by carbapenemase-producing isolates compared with isolates of the wild-type population (Stuart and Leverstein-Van Hall, 2010). The prevention of spread of carbapenemase producers relies on early and accurate detection of carriers in hospital units or on admission discharge either to the hospital or to a specific unit (Nordmann *et al.*, 2012a). Screening should include as a minimum ‘at-risk’ patients, such as those in intensive care units, transplant recipients and the immunocompromised, and those transferred

from any foreign hospital (unknown prevalence of carbapenemase producer carriage) or from non-foreign hospitals but known to face a high risk of carriage of carbapenemase producers (Nordmann and Poirel, 2012).

2.3.2.1.1. Screen agars

A medium initially designed to screen for extended spectrum beta-lactamase (ESBL) producers that contains carbapenem (CHROMagar KPC; CHROMagar Company, Paris, France (Reglier-Poupet *et al*., 2008). It detects carbapenem-resistant bacteria only if they exhibit high-level resistance to carbapenems (Nordmann *et al.*, 2012b). Its main disadvantage therefore remains its lack of sensitivity since it does not detect carbapenemase producers exhibiting a low level of carbapenem resistance, as observed for several MBL or OXA-48 producers (Carrër *et al*., 2010). The second screening medium also contains a carbapenem (CRE Brilliance, Thermo Fisher Scientific,UK) (Nordmann and Poirel, 2012). It detects KPC and MBL producers well, and most but not all OXA-48 producers (Nordmann and Poirel, 2012). The last and recently devloped screening medium , is a novel and patented medium (SUPERCARBA medium) containing cloxacillin, zinc and carbapenem molecule that has improved sensitivity and specificity for detecting all types of carbapenemase producer (including OXA-48 producers) (Nordmann *et al.*, 2012b).

2.3.2.2. Phenotypic detection of carbapenemase activity

2.3.2.2.1. Modified Hodge test (MHT)

The modified Hodge test (MHT) is a phenotypic screening test to identify carbapenemase producers, being recommended by the Clinical and Laboratory Standards Institute (CLSI) for Enterobacteriaceae with elevated carbapenem MICs or reduced disk diffusion inhibition zones (Pasteran *et al*., 2016). This test is based on the inactivation of a carbapenem by carbapenemase-producing strains, which enables a

susceptible indicator strain to extend growth toward a disk containing this antibiotic, along the streak of inoculum of the tested strain (Girlich *et al.*, 2012). The MHT has shown excellent sensitivity in the detection of class A and class D carbapenemase producers (Saito *et al*., 2015). Unfortunately, the MHT performs poorly in the detection of NDM-producing isolates, with sensitivity below 50% (Bonnin *et al*., 2012). Because NDMs are Zn (II)-dependent enzymes, it has been suggested that the deficits of this cation in commercial media could be responsible for these false-negative results (Girlich *et al.*, 2012). The MHT technique is highly sensitive for detecting class A, B, and D carbapenemases after addition of zinc in the culture medium and lack of specificity and the delay in obtaining the results (24 to 48 h) after isolation of a bacterial colony (Girlich *et al.*, 2012).

2.3.2.2.2. Synergy test

Synergy test is based on in vitro inhibition of carbapenemase activity by addition of an inhibitor specific for a class of carbapenemases (Khalil, 2018). For detection of class A carbapenemases, the inhibitor used is tazobactam, clavulanic acid or boronic acid (3-aminophenylboronic acid) (Nordmann and Poirel, 2012) and for detection of class B metallo carbapenemases, ethylene diamine tetra-acetic acid (EDTA), 2-mercaptopropionic acid, sodium mercaptoacetic acid or dipicolinic acid can be used as an inhibitor (Khalil, 2018).

2.3.2.2.3. Carba NP test

Carba NP testisa rapid and biochemical detection of carbapenemase production, which is based on the detection of the hydrolysis of the -lactam ring of imipenem (Poirel and Nordmann., 2015). The most important and recent development for the accurate identification of carbapenemase-producing *Enterobacteriaceae* is the Carba NP test (Nordmann and Poirel, 2012). It is based on *in vitro* hydrolysis of imipenem by a bacterial lysate, which is detected by the changes in pH values using the indicator phenol

red (red to yellow/orange) (Tijet *et al.*, 2013). This test is 100% sensitive and specific, as are molecular techniques (Nordmann and Poirel, 2012). It detects not only all known carbapenemases (belonging to Ambler A, B and D classes) in *Enterobacteriaceae* but should also identify virtually any new emerging 26 carbapenemase, in contrast to molecular techniques (Nordmann and Poirel, 2012). Overall, the Carba NP method was easy to perform, inexpensive and, in most cases, easy to interpret (Tijet *et al.*, 2013). This technique will soon become a reference technique since it fulfils the clinical requirement of a rapid and low-cost identification method for carbapenemase producing *Enterobacteriaceae* (Nordmann and Poirel, 2012).

2.3.2.3. Molecular detection of carbapenemases genes

Molecular techniques remain the gold standard for the precise identification of carbapenemase genes (Nordmann *et al.*, 2012a). Most of these techniques are based on PCR and may be followed by a sequencing step if a precise identification of the carbapenemase gene is needed (e.g. VIM type, KPC type, NDM type or OXA-48 type) (Nordmann and Poirel, 2012). They are either single or multiplex PCR techniques (Nordmann *et al.*, 2012a). A PCR technique performed directly on colonies can give results within 4–6 h (or less when using real-time PCR technology) with excellent sensitivity and specificity (Nordmann and Poirel, 2012). The main disadvantages of the molecular based technologies are their cost, the requirement for trained microbiologists and the inability to detect novel unidentified genes (Nordmann *et al.*, 2012a).

Sequencing of the genes is interesting mostly for research and epidemiological purposes (Nordmann and Poirel, 2012). Precise identification of the type of carbapenemase is not actually needed for treating patients or for preventing outbreaks (Nordmann *et al.*, 2012a).

These molecular techniques may be mostly used in reference laboratories (Nordmann and Poirel, 2012).

The DNA microarray may be more useful for epidemiological purposes or for infection control studies in case of high numbers of isolates have to be rapidly characterized (Dortet *et al.*, 2014b). Additionally, this procedure may also detect potential new carbapenemases (Dortet *et al.*, 2014b).

Colony blot hybridizations using labeled probes have been used to efficiently screen large numbers of clinical isolates for carbapenemase genes (Khalil, 2018).Southern blot hybridization too has been used to determine whether the carbapenemase gene resides on a plasmid or the chromosome (Khalil, 2018).

2.3.2.4 Previous studies

Mohammed *et al* (2015) in Nigeria characteried of KPC, NDM and VIM Type Carbapenem Resistance *Enterobacteriaceae* from North Eastern, Nigeria, they found that from 19 *Proteus mirabilis*, 2 (18.2%) harboring KPC and 1(50%) VIM.

Another study conducted by Demir and Karaoglan (2015) in Turkey they investigates of VIM, IMP, NDM-1, KPC AND OXA-48 enzymes in *Enterobacteriaceae* strains, they found that none of the *P. mirabilis* showed carbapenemase gene.

Mushi and his colleagues (2014) in Tanzania studied the Carbapenemase Genes among multidrug resistant gram negative clinical isolates. They found that none of the *P. mirabilis* showed carbapenemase gene.

Another study conducted by Okoshi and others (2015) in Uganda they studied the prevalence and Characterization of Carbapenem-Resistant Enterobacteriaceae Isolated from Mulago Mational Referral Hospital,they found that from 6 *P.mirabilis* isolates one have two genes (KPC,OXA-48).

Vourliand his colleagues (2006) in Greece studied the emergence of *P.mirabilis* carrying the blaVIM-1 metallo-β-lactamase gene. they found that seven *P.mirabilis* carrying the blaVIM-1 metallo-β-lactamase gene.

Another study conducted by Qin and others (2015) in China they characterized an NDM-1-producing clinical isolate of *P. mirabilis* (PM58) that displayed an extensively drug-resistant (XDR) phenotype.

Chen and his colleagues (2015) published the first report of an OXA-48-producing multidrug-resistant *P.mirabilis* strain in Gaza.

Tibbetts and his colleagues(2008) in Athens showed that the isolate of *P.mirabilis* recovered from blood cultures of a diabetic patient was shown to be resistant to imipenem, meropenem, and ertapenem by disk diffusion susceptibility testing. Amplification of whole-cell and/or plasmid DNA recovered from the isolate with primers specific for the *bla*KPC carbapenemase gene produced an amplicon of the expected size which was confirmed to be *bla*KPC-2 by sequence analysis.

Another study conducted by Ramos and his colleagues (2018) in Brazil they characterize multidrug-resistant *P.mirabilis* clones carrying a novel class 1 integron-borne *bla*IMP-1.

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study design

This study was a descriptive-case laboratory based study.

3.2. Study area

This study was conducted in Khartoum State. *P.mirabilis* isolates were collected from the following hospitals; Omdurman Military Hospital, Aliaa Hospital, Soba University Hospital. The study was performed at Sudan University of Science and Technology, College of Medical Laboratory Science.

3.3. Study duration

The study was conducted during the period from March to November 2019.

3.4 Study samples

The isolates of *P.mirabilis* were obtained from laboratory of hospitals, which previously isolated from clinical specimens (urine, wound swabs, high vaginal swabs and semen).

3.5. Ethical considerations

The study was approved by Research Board of College of Medical Laboratory Science, Sudan University of Science and Technology.

3.6. Sampling technique

Non-probability convenience sampling technique.

3.6.1. Sample size

A total of fifty *P.mirabilis* isolates (n=50) were collected for this study

.3.7. Data collection

Data was collected from hospital check list.

3.8. Laboratory Methods

3.8.1. Samples preservation

The isolated organisms were stored in Research Laboratory in Sudan University of Science and Technology, in 20% Glycerol peptone media.

3.8.2. Subculture

Few drops of glycerol peptone media containing organism were inoculated on Nutrient agar and MacConkey's agar and incubated overnight at 37°C aerobically.

3.8.3. Reidentification of isolates

3.8.3.1 Gram stain

A Primary stains "Crystal violet‖ was applied to the dry–heat–fixed smear of microorganism for1minute.Then the stain was washed with Water and cover with Lugol'siodinefor1minute.Stain was washed with distilled water. And decolorized by acetone, alcohol and washed with distilled water. Then the stain was covered with safarnin for 2minutes.The slide was placed in a rack to dry. The specimen was examined at (X100) (Oil-immersionlens) (Cheesbrough, 2006).

3.8.3.2 Biochemical tests

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 (Kligler Iron Agar, Tryptophan peptone water, Simmon′s citrate agar, Christensen′s urea agar) (HIMEDIA, India), and then incubated at 37°C in an incubator, then interpreted according to their reactions (Cheesbrough, 2006).

3.8.3.2.1 Indole test

A sterile loop was used to inoculate the tested organisms into (2 ml) peptone water; the tube was incubated at (37°C) for (24 hrs) in an incubator. In the next day, add drop of Kovac′s reagent (4-dimethylamino benzaldehyde), it was shacked gently and examined for red color ring within (10 mints). *P.mirabilis* is negative for indole test (Cheesbrough, 2006).

3.8.3.2.2 Kligler Iron Agar (KIA)

The tested organisms were 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) in an incubator. Glucose fermentation, lactose fermentation, H2S production and gas production were reported. *P.mirabilis* gave yellow butt and red slope, H2S production and no gas production (Cheesbrough, 2006).

3.8.3.2.3 Urease test

The tested organisms were 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 inculcated and incubated overnight at (37°C) in an incubator, *P.mirabilis* is urease positive (produce pink color) (Cheesbrough, 2006).

3.8.3.2.4 Citrate utilization test

The tested organisms were picked up by a straight wire and inoculated in sloped Simmon's citrate agar and incubated overnight at 37°C in an incubator, after incubation the media was tested for change in color, *P.mirabilis* gave positive result (blue color) (Cheesbrough, 2006).

3.8.4. Susceptibility testing

Kirby Bauer CLSI modified disc diffusion technique, Mueller Hinton agar was used for antimicrobial susceptibility testing. By using a sterile wire loop, 3-5 colonies of similar appearance of the tested organism were picked and emulsified in 3-4 ml of sterile physiological saline, and were matched with 0.5% McFarland standard in a good light. Then sterile swab was used to inoculate a plate of Mueller Hinton agar, excess fluid was removed and swab was streaked evenly over the surface of the medium in three directions to ensure even distribution, and then allowed 3-5 minute to dry. Using sterile forceps, needle mounted in a holder or a multidisc dispenser, the appropriate antimicrobial discs were placed and distributed evenly on

the inoculated plate. After incubation at 37˚C overnight, the zones diameters were recorded and interpreted according to CLSI guidelines. Ceftazidime (30mcg), ciprofloxacin (5mcg), amikacin (30mcg) ،gentamicin (10mcg) and imipenem (10mcg) were used in this study with the control strain *Escherichia coli* ATCC 25922.

3.8.5. Detection of carbapenem resistance genes of *P.mirabilis*

3.8.5.1. DNA extraction

DNA extraction with GenoLyse procedure. All scraped colonies were washed with 500µl normal saline (NS), then centerfuge for 15 minutes at 10.000 rpm, discarded supernatant and added 100µl lysis buffer, then resuspended and incubated for 5 minute at 95°C, then add 100µl neutralization buffer and vortex, then centrifuge for 5 minute at full speed and supernatant was taken new tube. The DNA samples were stored at-20°C until used for PCR (Hain Life Sciences, Germany).

3.8.5.2. PCR technique

3.8.5.2.1. Primers

Primers set were used to detect carbapenem resistant genes of *P.mirabilis*. The primers were dissolved according to manufacture guidelines to prepare 10 pmol /µL.

Table 3.1 Primers sequences and amplicons size used for detection of carbapenem resistance genes of *P.mirabilis*

Primer name	DNA sequence (5' to 3')	Amplicons size (bp)
KPC	Forward: CATTCAAGGGCTTTCTTGCTGC Reverse: ACGACGGCATAGTCATTTGC	498
NDM	Forward: GGTTTGGCGATCTGGTTTTC Reverse: CGGAATGGCTCATCACGATC	521
IMP	Forward: TTGACACTCCATTTACAG Reverse: GATTGAGAATTAAGCCACTCT	232
OXA-48	Forward: GCTTGATCGCCCTCGATT Reverse: GATTTGCTCCGTGGCCGAAA	281
VIM	Forward: GATGGTGTTTGGTCGCATA Reverse: CGAATGCGCAGCACCAG	390

3.8.5.2.2. Preparation of 10X Tris Base EDTA (TBE) buffer

An amount of 48.4g of Tris base was added to 55g of boric acid and 7.44g of EDTA and then dissolved into 1L of D.W.

3.8.5.2.3. Preparation of 1X TBE buffer

10ml of 10X were added to 90ml D.W and mixed.

3.8.5.2.4. Preparation of ethidium bromide

10mg of ethidium bromide were dissolved into 0.5ml D.W and kept into brown bottle.

3.8.5.2.5. Preparation of agarose gel

Amount of 1.5 g of agarose powder were dissolved into 100 ml of 1X TBEusing microwave for 30s. The mixture had been cooled and then 2µl of ethidium bromide was added ،mixed well and poured in a casting tray that had been taped up appropriately and equipped with spacers and stable comb to form wells. Any bubbles were removed and the gel allowed setting

at room temperature, after solidification, the comb and the spacers were gently removed.

3.8.5.2.6. Master Mix

Maxime PCR PreMix kit (iNtRON Biotechnology, Seongnam, Korea) was used.

3.8.5.2.7. Preparation of reaction mixtures

3.8.5.2.7.1. Reaction mixture for carbapenem resistance genes

Multiplex PCR was carried out in a 20µl volume using the Maxime PCR PreMix kit (iNtRON Biotechnology, Seongnam, Korea), premix were dissolved by 16 µl of D.W, transferred in to 0.5 ml PCR tube, for each gene 0.4 µl of forward primer, 0.4 µl of reverse primer, 2 µl of DNA were added.

3.8.5.2.8. Amplification Protocol of NDM, IMP and VIM

The PCR was done by using a thermocycler (Convergys, Germany) for amplification with the following conditions: initial activation at 94°C for 2 minutes, followed by 35 cycles at 94°C for 20s, 56°C for 10s, and 72°C for 20s, and a final extension at 72°C for 5 minutes.

3.8.5.2.8.1. Amplification Protocol of KPC and OXA-48 genes

The PCR was done by using a thermocycler (Convergys, Germany) with the following conditions: initial activation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 45s, 52°C for 1 minute, and 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

3.8.5.2.9. Visualization of PCR product

The amplicons were separated at 50V for 1hr in a 1.5% (wt/vol) agarose gel containing ethidium bromide, bands were visualized under U.V transilluminator (UVitec–UK) to detect the specific amplified products by comparing with 100bp standard ladder (INtRON biotechnology. Korea).

3.9. Data analysis and presentation

The data obtained were analyzed and presented using Statistical Package for Social Science (SPSS) computer software version 16.0 for Windows. Results were presented in form at tables and figures.

CHAPTER FOUR

RESULTS

CHAPTER FOUR

RESULTS

4.1. Distribution of *P.mirabilis* isolates according to the type of specimen

Out of fifty *P.mirabilis* isolates, 21 (42%) were isolated from urine, 26 (52%) from wound swabs, 2(4%) from high vaginal swabs and 1(2%) from semen specimen (Figure 4.1).

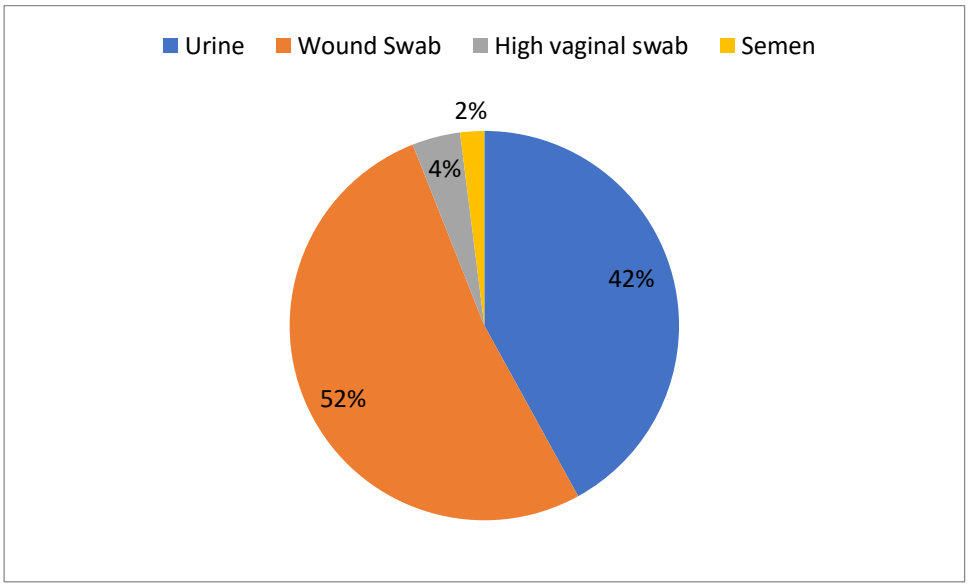


Figure (4.1): Distribution of *P.mirabilis* according to the type of specimen

4.2. Results of Antibiotics susceptibility test

Out of fifty isolates, 48(96%) were resistant to ceftazidime,31(62%) were resistant to ciprofloxacin, 30(60%) were resistant to amikacin, 43(86%) were resistant to gentamicin and 13(26%) were resistant to imipenem.

Table (4.1): Results of antibiotics susceptibility test

Antibiotics Results	Sensitive	Resistant
Ceftazidime (30mcg)	2(4%)	48(96%)
Ciprofloxacin (5mcg)	19(38%)	31(62%)
Amikacin (30mcg)	20(40%)	30(60%)
Gentamicin (10mcg)	7(14%)	43(86%)
Imipenem (10mcg)	37(74%	13(26%)

4.3. Detection of *P.mirabilis* carbapenem resistance genes

All 50 *P.mirabilis* isolates investigated for the presence of carbapenem resistance genes (NDM, IMP, VIM, OXA-48 and KPC) using multiplex PCR. All *P.mirabilis* isolates were negative 0(0%) for all carbapenem five resistance genes (Figure 4.2).

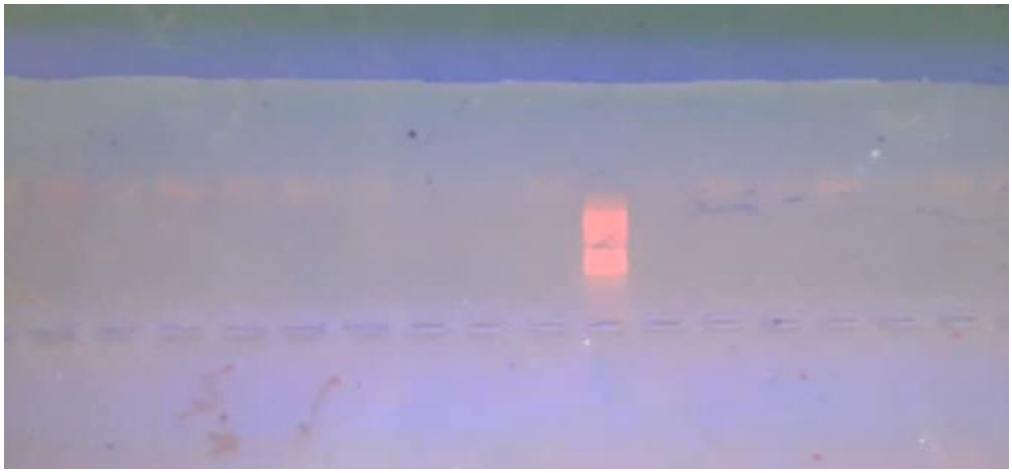


Figure (4.2): Gel electrophoresis of carbapenem resistance genes (NDM, IMP, VIM, OXA-48 and KPC)

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. Discussion

The emergence and spread of carbapenem resistance *Enterobacteriaceae* (CRE) have become an increasing concern for healthcare services worldwide, especially when mediated by transferable carbapenem seen coding genes (Baran and Aksu, 2016). Infections caused by these bacteria have been associated with significant morbidity and mortality and treatment options have been limited (Baran and Aksu, 2016).

In this study, 50 *P. mirabilis* isolates were collected from different hospitals in Khartoum State and were cultured on nutrient agar and MacConkey's agar. All isolates were identified using biochemical tests.

Multiplex PCR was used to detect five carbapenem resistant genes (NDM, IMP, VIM, OXA-48 and KPC).

The present study demonstrated that from 50 *P. mirabilis* isolates 48(96%) were resistant to ceftazidime. The result of present study agree with the study of Mushi *et al* (2014) in Tanzania who reported that (100%) of *P. mirabilis* were resistant to ceftazidime. Different results were reported by Hashemi *et al* (2013), from west of Iran who found that (70%) of *P. mirabilis* were resistant to ceftazidime.

In present study (62%) of *P. mirabilis* were resistant ciprofloxacin. These results were contrast to the studies of Al-Jumaily and Zgaer (2016) in Baghdad City who reported that (33%) of *P. mirabilis* were resistant to Ciprofloxacin.

In this study (60%) of *P. mirabilis* were resistant to amikacin. This is slightly similar to the study of Tsai and his collegues (2014) in Taiwan they reported that (46%) of *P. mirabilis* were resistant to amikacin. In the present study (86%) of *P. mirabilis* were resistant to gentamicin. This

result dis agree with study of Hussein (2018) who reported that (60%) of *P. mirabilis* were resistant to gentamicin.

The result of present study demonstrated that (26%) of *P. mirabilis* were resistant to imipinem.This result is nearly similar to the study of Nazeih and Abbas (2019**)** in Zagazig whom reported that (15%) of *P. mirabilis* were resistant to imipenem and differ from the study of Zafar and his collegues (2019) in Quetta who reported that (96%) of *P. mirabilis* were resistant to imipenem.

In this study, *P. mirabilis* isolates were negative for all five genes studied. This result is comparable to study of Mushi *et al* (2014) in Tanzania and differ from the study of Vourliand his colleagues (2006) in Greece they found that seven *P.mirabilis* carrying blaVIM-1 metallo-β-lactamase gene and also disagree with study of Ramos and his colleagues (2018) in Brazil they characterize multidrug-resistant *P.mirabilis* clones carrying a novel class 1 integron-borne *bla*IMP-1.

In this study, *P.mirabilis* isolates were negative for all five genes in spite of resistance to antibiotics, this due to these bacteria may contain carbapenem resistance genes other than genes included in this study such as: SPM, SME, NMC-A, IMI-2…etc, also these bacteria may have other mechanisms of resistance such as drug efflux and porin loss.

5.2. Conclusion

According to sample collection this study concluded that there is no carbapenem resistance genes (NDM, IMP, VIM, OXA-48 and KPC) among *P.mirabilis* isolates in Khartoum State.

5.3. Recommendations

This study recommended that:

Large sample size to be studied to confirm these results.

Study of other carbapenem resistant genes such as SPM, NMC-A,SPM,…etc.

It is of great importance to make a unique and precise guideline for routine Microbiology Laboratories in order to detect carbapenemase producing isolates.

Adhering to extensive infection control measures in community and hospital-acquired infections to disrupt the further spread of carbapenemase-producing isolates among hospitalized patients in Sudan.

Apply sensitivity testing to other carbapenem as meropenem, ertapenem.

REFERENCES

Ali, H.H. and Yousif, M.G., (2015). Detection of some virulence factors genes of *Proteus mirablis* that isolated from urinary tract infection. *IJAR*, **3**(1), pp.156-163.

Al-Jumaily, E. and Zgaer, S., (2016). Multidrug resistant *Proteus mirabilis* isolated from urinary tract infection from different hospitals in Baghdad City. *Int. J. Curr. Microbiol. App. Sci*, **5**(9), pp.390-399.

Al-Kazaz, A.K. and Al-Bassam, W.W., (2013). The isolation and characterization of *Proteus mirabilis* from different clinical samples. *Jornal of Biotechnology Research Center*, **7**(2), pp.24-30.

Armbruster, C.E., Mobley, H.L. and Pearson, M.M., (2018). Pathogenesis of *Proteus mirabilis* infection. *EcoSal Plus*, **8**(1).

Asif, M.,Alvi, I.A. and Rehman, S.U., (2018). Insight into Acinetobacter baumannii: pathogenesis, global resistance, mechanisms of resistance, treatment options, and alternative modalities. *Infection and drug resistance*, 11, p.1249.

Baran, I. and Aksu, N. (2016). Phenotypic and genotypic characteristics of carbapenem-resistant *Enterobacteriaceae* in a tertiary-level reference hospital in Turkey. *Annals of Clinical Microbiology and Antimicrobials*, **15**(1).

Bassetti, M. Nicolini, L., Esposito, S., Righi, E. and Viscoli, C., (2009). Current status of newer carbapenems. *Current medicinal chemistry*, **16**(5), pp.564-575.

Bedenić, B. Plečko, V., Sardelić, S., Uzunović, S. and GodičTorkar, K., (2014). Carbapenemases in gram-negative bacteria: laboratory detection and clinical significance. *BioMed research international, 2014*.

Bonnin, R.A., Naas, T., Poirel, L. and Nordmann, P.,(2012). Phenotypic, biochemical, and molecular techniques for detection of metallo-β-

lactamase NDM in Acinetobacter baumannii. *Journal of clinical microbiology*, **50**(4), pp.1419-1421.

Carrër, A., Fortineau, N. and Nordmann, P., (2010). Use of ChromID extended-spectrum β-lactamase medium for detecting carbapenemase-producing Enterobacteriaceae. *Journal of clinical microbiology*, **48**(5), pp.1913-1914.

Cheesbrough, M. (2006). District laboratory practice in tropical countries, part 2. Cambridge university press.

Chen, L., Al Laham, N., Chavda, K.D., Mediavilla, J.R., Jacobs, M.R., Bonomo, R.A. and Kreiswirth, B.N., (2015). First report of an OXA-48-producing multidrug-resistant *Proteus mirabilis* strain from Gaza, Palestine. *Antimicrobial agents and chemotherapy*, **59**(7), pp.4305-4307.

Coulthurst, S.J., Barnard, A.M. and Salmond, G.P., (2005). Regulation and biosynthesis of carbapenem antibiotics in bacteria. *Nature Reviews Microbiology*, **3**(4), p.295.

da Silva, G.J. and Mendonça, N., (2012). Association between antimicrobial resistance and virulence in *Escherichia c*oli. *Virulence*, **3**(1), pp.18-28.

Dahiya, S., Singla, P., Chaudhary, U. and Singh, B., (2015). Carbapenemases: a review. *Int J Adv Health Sci*, **2**(4), pp.11-17.

Demir, Y., Zer, Y. and Karaoglan, I., (2015). Investigation of VIM, IMP, NDM-1, KPC AND OXA-48 enzymes in Enterobacteriaceae strains. *Pakistan journal of pharmaceutical sciences*, *28*.

Di Pilato, V., Chiarelli, A., Boinett, C.J., Riccobono, E., Harris, S.R., D'Andrea, M.M., Thomson, N.R., Rossolini, G.M. and Giani, T., (2016). Complete genome sequence of the first KPC-type carbapenemase-positive *Proteus mirabilis* strain from a bloodstream infection. *Genome Announc.*, **4**(3), pp.e00607-16.

Dortet, L.,Poirel, L. and Nordmann, P. (2014a). WorldwideDissemination of the NDM-Type Carbapenemases in Gram-Negative bacteria. *BioMed Research International*, pp.1-12.

Findlay, J., Hopkins, K.L., Doumith, M., Meunier, D., Wiuff, C., Hill, R., Pike, R., Loy, R., Mustafa, N., Livermore, D.M. and Woodford, N., (2016). KPC enzymes in the UK: an analysis of the first 160 cases outside the North-West region. *Journal of Antimicrobial Chemotherapy*, **71**(5), pp.1199-1206.

Fursova, N.K.,Astashkin, E.I., Knyazeva, A.I., Kartsev, N.N., Leonova, E.S., Ershova, O.N., Alexandrova, I.A., Kurdyumova, N.V., Sazikina, S.Y., Volozhantsev, N.V. and Svetoch, E.A., (2015). The spread of bla OXA-48 and bla OXA-244 carbapenemase genes among *Klebsiella pneumoniae*, *Proteus mirabilis* and *Enterobacter spp*. isolated in Moscow, Russia. *Annals of clinical Microbiology and Antimicrobials*, **14**(1), p.46.

Gharrah, M., Mostafa El-Mahdy, A. and Barwa, R. (2017). Association between Virulence Factors and Extended Spectrum Beta-LactamaseProducing *Klebsiella pneumoniae* Compared to Nonproducing Isolates. *Interdisciplinary Perspectives on Infectious Diseases*, pp.1-14.

Girlich, D., Poirel, L. and Nordmann, P. (2012). Value of the Modified Hodge Test for Detection of Emerging Carbapenemasesin *Enterobacteriaceae*. *Journal of Clinical Microbiology*, **50**(2), pp.477-479.

Guducuoglu, H., Gursoy, N.C., Yakupogullari, Y., Parlak, M., Karasin, G., Sunnetcioglu, M. and Otlu, B., (2018). Hospital outbreak of a colistin-resistant, NDM-1-and OXA-48-producing *Klebsiella pneumoniae*: high mortality from pandrug resistance. *Microbial Drug Resistance*, **24**(7), pp.966-972.

Hasan, A.R.S., Nauman, N.G. and Al-Duliami, A.A., (2011). Virulence Factors of *Proteus mirabilis* Isolated From Patients Otitis Media in Baquba And it's Peripheries. *Diyala Journal of Medicine*, **1**(1), pp.69-75.

Hashemi, S. H., ESNA, A. F., Tavakoli, S., &Mamani, M. (2013). The prevalence of antibiotic resistance of enterobacteriaceae strains isolated in community-and hospital-acquired infections in teaching hospitals of Hamadan, west of Iran**.**

Hawkey, P.M. and Jones, A.M., (2009). The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy*, **64**(suppl_1), pp.i3-i10.

Hu, Y.Y., Cai, J.C., Zhang, R., Zhou, H.W., Sun, Q. and Chen, G.X.,(2012). Emergence of *Proteus* mirabilis harboring blaKPC-2 and qnrD in a Chinese hospital. *Antimicrobial agents and chemotherapy*, **56(**5), pp.2278-2282.

Hussein, E. F. (2018). Detection of Antibiotics Sensitivity at different pH levels for *Proteus mirabilis* Isolated from patients with Urinary Tract Infections. *Journal of University of Babylon for Pure and Applied Sciences*, **26**(5), 132-144.

Jacobsen, S.M., D.J. Stickler, H.L.T. Mobley, and M.E. Shirtliff. (2008). Complicated catheter-associated urinary tract infections due to *Escherichia coli* and *Proteus mirabilis*. Clin. Microbiol. Rev. **21**(1): 26 59.

Jacobsen, S. M., & Shirtliff, M. E. (2011). Proteus mirabilis biofilms and catheter-associated urinary tract infections. *Virulence*, **2**(5), 460-465.

Jeon, J., Lee, J., Lee, J., Park, K., Karim, A., Lee, C.R., Jeong, B. and Lee, S.,(2015). Structural basis for carbapenem-hydrolyzing mechanisms of carbapenemases conferring antibiotic resistance. *International journal of molecular sciences*, **16**(5), pp.9654-9692.

Khalil,S. A. A. I. (2018). *Molecular Detection of Carbapenem Resistance Genes of Klebsiella pneumoniae Isolated from Clinical Specimens in Khartoum State* (Doctoral dissertation, Sudan University of Science & Technology).

Lee, C.R., Lee, J.H., Park, K.S., Kim, Y.B., Jeong, B.C. and Lee, S.H., (2016). Global dissemination of carbapenemase-producing *Klebsiella pneumoniae:* epidemiology, genetic context, treatment options, and detection methods. *Frontiers in microbiology*, 7, p.895.

Liaw, S.J., Lai, H.C. and Wang, W.B.,(2004). Modulation of swarming and virulence by fatty acids through the RsbA protein in *Proteus mirabilis. Infection and immunity*, **72**(12), pp.6836-6845.

Lutgring, J.D. and Limbago, B.M.,(2016).The problem of carbapenemase-producing-carbapenem-resistant-Enterobacteriaceae detection. *Journal of clinical microbiology*, **54**(3), pp.529-534.

Manos, J. and Belas, R., (2006). The genera Proteus, *Providencia*, and *Morganella*. *The Prokaryotes: Volume 6: Proteobacteria: Gamma Subclass*, pp.245-269.

Meletis, G. (2016). Carbapenem resistance: overview of the problem andfuture perspectives. *Therapeutic Advances in Infectious Disease*, **3**(1),pp.15-21.

Miriagou, V., Cornaglia, G., Edelstein, M., Galani, I., Giske, C.,Gniadkowski, M., Malamou-Lada, E., Martinez-Martinez, L., Navarro, F., Nordmann, P., Peixe, L., Pournaras, S., Rossolini, G., Tsakris, A., Vatopoulos, A. and Cantón, R. (2010). Acquired carbapenemases in Gramnegative bacterial pathogens: detection and surveillance issues. *Clinical Microbiology and Infection*, **16**(2), pp.112-122.

MoelleringJr, R.C., (2010). NDM-1—a cause for worldwide concern. *New England Journal of Medicine*, **363**(25), pp.2377-2379.

Mohammed, Y., Zailani, S.B. and Onipede, A.O., (2015). Characterization of KPC, NDM and VIM type carbapenem resistance Enterobacteriaceae from North Eastern, Nigeria. *Journal of Biosciences and Medicines*, **3**(11), p.100.

Munoz-Price, L. S.,& Quinn, J. P. (2009). The spread of *Klebsiella pneumoniae* carbapenemases: a tale of strains, plasmids, and transposons.

Musa, H.A., Osman, M.A., Abdelaziz, Y.H., Mohamed, S. and Ibrahim-Saeed, M., (2019). Distribution of extended-spectrum beta-lactamase TEM and CTX-M resistance genes among *Proteus* species isolated in Sudan. *VacciMonitor*, **28**(2), pp.80-84.

Mushi, M.,Mshana, S., Imirzalioglu, C. and Bwanga, F. (2014). Carbapenemase Genes among Multidrug Resistant Gram Negative ClinicalIsolates from a Tertiary Hospital in Mwanza, Tanzania. *BioMed ResearchInternational*, pp.1-6.

Nazeih, S. I., Serry, F., and Abbas, H. (2019). Study on increased antimicrobial resistance among bacteria isolated from ICUs Zagazig University Hospitals. *Zagazig Journal of Pharmaceutical Sciences*, **28**(1), 13-25.

Nicoletti, G., Russo, G. and Bonfiglio, G.,(2002). Recent developments in carbapenems. *Expert opinion on investigational drugs*, **11**(4), pp.529-544.

Nordmann, P. and Poirel, L. (2012). Strategies for identification of carbapenemase-producing *Enterobacteriaceae*. *Journal of Antimicrobial Chemotherapy*, **68**(3), pp.487-489.

Nordmann, P.,Cuzon, G. and Naas, T., (2009). The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *The Lancet infectious diseases*, **9**(4), pp.228-236.

Nordmann, P., Girlich, D. and Poirel, L. (2012b). Detection of Carbapenemase Producers in *Enterobacteriaceae* by Use of a Novel 58Screening Medium. *Journal of Clinical Microbiology*, **50**(8), pp.2761-2766.

Nordmann, P., Gniadkowski, M., Giske, C., Poirel, L., Woodford, N. and Miriagou,V.(2012a).Identification and screening of carbapenemase

producing *Enterobacteriaceae*. *Clinical Microbiology and Infection*, **18**(5), pp.432-438.

Nordmann, P., Poirel, L., Walsh, T.R. and Livermore, D.M., (2011). The emerging NDM carbapenemases. *Trends in microbiology*, **19**(12), pp.588-595.

Okoche, D., Asiimwe, B.B., Katabazi, F.A., Kato, L. and Najjuka, C.F., (2015). Prevalence and characterization of carbapenem-resistant Enterobacteriaceae isolated from Mulago National Referral Hospital, Uganda. *PLoS One*, **10**(8), p.e0135745.

Pal, N., Sharma, N., Sharma, R., Hooja, S., & Maheshwari, R. K. (2014). Prevalence of multidrug (MDR) and extensively drug resistant (XDR) Proteus species in a tertiary care hospital, India. *Int J Curr Microbiol Appl Sci*, *3*, 243-52.

Papp-Wallace, K.M.,Endimiani, A., Taracila, M.A. and Bonomo, R.A., (2011). Carbapenems: past, present, and future. *Antimicrobial agents and chemotherapy*, **55**(11), pp.4943-4960.

Pasteran, F., Gonzalez, L.J., Albornoz, E., Bahr, G., Vila, A.J. and Corso, A., (2016). Triton Hodge test: improved protocol for modified Hodge test for enhanced detection of NDM and other carbapenemase producers. *Journal of clinical microbiology*, **54**(3), pp.640-649.

Patel, G. and Bonomo, R.A., (2011). Status report on carbapenemases: challenges and prospects. *Expert review of anti-infective therapy*, **9**(5), pp.555-570.

Poirel, L. and Nordmann, P., (2015). RapidecCarba NP test for rapid detection of carbapenemase producers. *Journal of clinical microbiology*, **53**(9), pp.3003-3008.

Poirel, L., Naas, T. and Nordmann, P., (2010). Diversity, epidemiology, and genetics of class D β-lactamases. *Antimicrobial agents and chemotherapy*, **54**(1), pp.24-38.

porin,Girlich, D., Dortet, L., Poirel, L. and Nordmann, P., (2014). Integration of the bla NDM-1 carbapenemase gene into Proteus genomic island 1 (PGI1-Pm PEL) in *a Proteus mirabilis* clinical isolate. *Journal of Antimicrobial Chemotherapy*, **70**(1), pp.98-102.

Qin, S., Qi, H., Zhang, Q., Zhao, D., Liu, Z.Z., Tian, H., Xu, L., Xu, H., Zhou, M., Feng, X. and Liu, H.M., 2015. Emergence of extensively drug-resistant *Proteus* mirabilis harboring a conjugative NDM-1 plasmid and a novel Salmonella genomic island 1 variant, SGI1-Z. *Antimicrobial agents and chemotherapy*, **59**(10), pp.6601-6604.

Queenan, A.M. and Bush, K., (2007). Carbapenemases: the versatile β-lactamases. *Clinical microbiology reviews*, **20**(3), pp.440-458.

Ramos, A.C., Cayô, R., Carvalhaes, C.G., Jové, T., da Silva, G.P., Sancho, F.M.P., Chagas-Neto, T., Medeiros, E.A.S. and Gales, A.C., (2018). Dissemination of multidrug-resistant Proteus mirabilis clones carrying a novel integron-borne blaIMP-1 in a tertiary hospital. *Antimicrobial agents and chemotherapy*, **62**(2).

Rasmussen, J. and Høiby, N., (2007). Class A carbapenemases. *Journal of Antimicrobial Chemotherapy*, **60**(3), pp.470-482.

Reglier-Poupet, H., Naas, T., Carrer, A., Cady, A., Adam, J.M., Fortineau, N., Poyart, C. and Nordmann, P., (2008). Performance of chromID ESBL, a chromogenic medium for detection of Enterobacteriaceae producing extended-spectrum β-lactamases. *Journal of medical microbiology*, **57**(3), pp.310-315.

Różalski, A., Torzewska, A., Moryl, M., Kwil, I., Maszewska, A., Ostrowska, K., ... & Staçzek, P. (2012). Proteus sp.–an opportunistic bacterial pathogen–classification, swarming growth, clinical significance and virulence factors. *Folia Biologica et Oecologica*, **8**(1), 1-17.

Saito, R.,Koyano, S., Dorin, M., Higurashi, Y., Misawa, Y., Nagano, N., Kaneko, T. and Moriya, K., (2015). Evaluation of a simple phenotypic method for the detection ofcarbapenemase- Producing Enterobacteriaceae. *Journal of microbiological methods*, 108, pp.45-48.

Senchyna, F., Gaur, R., Sandlund, J., Truong, C., Tremintin, G., Küeltz, D., Gomez, C., Tamburini, F.B., Andermann, T.M., Bhatt, A.S. and Tickler, I.A.,(2018). Diverse Mechanisms of Resistance in Carbapenem-Resistant Enterobacteriaceae at a Health Care System in Silicon Valley, California. *bioRxiv*, p.298513.

Shen, P., Wei, Z., Jiang, Y., Du, X., Ji, S., Yu, Y. and Li, L.,(2009). Novel genetic environment of the carbapenem-hydrolyzing β-lactamase KPC-2 among Enterobacteriaceae in China. *Antimicrobial agents and chemotherapy*, **53(**10), pp.4333-4338.

Stuart, J.C. and Leverstein-Van Hall, M.A.,(2010). Guideline for phenotypic screening and confirmation of carbapenemases in Enterobacteriaceae. *International journal of antimicrobial agents*, **36**(3), pp.205-210.

Tijet, N., Boyd, D., Patel, S. N., Mulvey, M. R., &Melano, R. G. (2013). Evaluation of the Carba NP test for rapid detection of carbapenemase-producing Enterobacteriaceae and Pseudomonas aeruginosa. *Antimicrobial agents and chemotherapy*, **57**(9), 4578-4580.

Tibbetts, R., Frye, J.G., Marschall, J., Warren, D. and Dunne, W., (2008). Detection of KPC-2 in a clinical isolate of *Proteus mirabilis* and first reported description of carbapenemase resistance caused by a KPC β-lactamase in P. mirabilis. *Journal of clinical microbiology*, **46**(9), pp.3080-3083.

Tsai, H.Y., Chen, Y.H., Tang, H.J., Huang, C.C., Liao, C.H., Chu, F.Y., Chuang, Y.C., Sheng, W.H., Ko, W.C. and Hsueh, P.R.,(2014). Carbapenems and piperacillin/tazobactam for the treatment of bacteremia

caused by extended-spectrum β-lactamase–producing *Proteus mirabilis*. *Diagnostic microbiology and infectious disease*, **80** (3), pp.222-226.

Vourli, S., Tsorlini, H., Katsifa, H., Polemis, M., Tzouvelekis, L.S., Kontodimou, A. and Vatopoulos, A.C., (2006). Emergence of *Proteus mirabilis* carrying the blaVIM-1 metallo-β-lactamase gene. *Clinical microbiology and infection*, 12(7), pp.691-694.

Walsh, T.,Toleman, M., Poirel, L. and Nordmann, P. (2005). Metallo- β-Lactamases: the Quiet before the Storm?.*Clinical Microbiology Reviews*,**18**(2), pp.306-325.

Wang, W.B., Lai, H.C., Hsueh, P.R., Chiou, R.Y.Y., Lin, S.B. and Liaw, S.J.,(2006). Inhibition of swarming and virulence factor expression in *Proteus mirabilis* by resveratrol. *Journal of medical microbiology*, **55**(10), pp.1313-1321.

Wu, W., Feng, Y., Tang, G., Qiao, F., McNally, A. and Zong, Z., (2019). NDM metallo-β-lactamases and their bacterial producers in health care settings. *Clinical microbiology reviews*, **32**(2), pp.e00115-18.

Zafar, U., Taj, M. K., Nawaz, I., Zafar, A., & Taj, I. (2019). Characterization of *Proteus mirabilis* Isolated from Patient Wounds at Bolan Medical Complex Hospital, Quetta. *Jundishapur Journal of Microbiology*, **12**(7), 1-6.

Zhanel, G. G., Wiebe, R., Dilay, L., Thomson, K., Rubinstein, E., Hoban, D. J., ... &Karlowsky, J. A. (2007). Comparative review of the carbapenems. *Drugs*, **67**(7), 1027-1052.

APPENDICES

Appendix I:

Equipments:

Microscope

Autoclave

Incubator

Microwave

Thermocycler

Water bath

Vortex

UV light

Appendix II:

Reagents and culture media

1: Crystal violet (HilMedia Laboratories Pvt. Ltd. Mumbai, Inida)

Ingredients g/L

Crystal violet 20g

Ammonium oxalate 9 g

Ethanol or methanol, absolute 95 ml.

Preparation:

Weigh the crystal violet on a piece of clean paper (pre weighed), transfer to a brown bottle, pre marked to hold I liter, add the absolute ethanol or methanol (technical grade is suitable) and mix until the dye is completely dissolved, weight the ammonium oxalate and dissolve in about 200 ml of distilled water, add to the stain, make up to the I liter mark with distilled water, and mix well (Caution: Ammonium oxalate is a toxic chemical, there fore handle it with care), label the bottle, and store it at room temperature. The stain is stable for several months.

2: Lugol's iodine (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients g/L

Potassium iodide

Iodine 10 g.

Preparation:

Weight the Potassium iodide, and transfer to a brown bottle pre marked to hold 1 liter, add about a quarter of the volume of water, and mix until the potassium iodide is completely dissolved, weight the iodine, and add to the potassium iodide solution. Mix until the iodine is dissolved (Caution: iodine is injurious to health if inhaled or allowed to come in contract with eyes., therefore handl eit with care in a well ventilated room and make up to the I liter mark with distilled water, and mix well. Label the bottle, and mark it Toxic. Store it in a dark place at room temperature Renew the solution if it's colour fades.

3: Acetone-alcohol decolorize (HI Media Laboratories Pvt. Ltd. Mumbai, India)

.Ingredients g/L

Acetone 500Ml

Ethanol or methanol, absolute 475 Ml

Preparation:

Mix the distilled water with the absolute ethanol (ethyl alcohol) or methanol (methy1 alcohol), transfer the solution to a screw-cap bottle of 1 liter capacity, technical grade is adequate, measure the acetone, and add immediately to the alcohol solution mix well (Caution: Acetone is a highly flammable chemicalthat vaporizes rapidly, therefore use it well away from an open flame) and labelthe bottle, and mark it Highly flammable. Store in a safe place at room temperature the reagent is stable indefinitely.

4: Safranin (HiMedial Laboratories Pvt. Ltd. Mumbai, India)

Ingredients g/L

Safranin O 0.50

Ethyl alcohol, 95% 100.11

5: Eosin Methylene Blue —HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients g/L

Peptone 10.000

Lactose 10.000

Di postassuim Hydrogen phosphate 3.000

Eosin yellow dye 0.400

Methylene Blue dye 0.065

Agar 14.000

Directions:

Suspend 37.5 g in 1000 ml of cold distilled water. Heat to boiling, string constantly, distribute and autoclave at 121 °C for 15 min cool to about 60°C and before transferring to plates gently shake the flask to oxidize the medium and 46 to disperse the flocculent precipitate that forms darning sterilization final PH 7.1 /+ -0.2.

6: Kligler Iron Agar (HiMedia Laboratories Pvt. Ltd. Mumbai, Ingredients India).

Ingredients g/L

Peptic digest of animal tissue 15.00

Beef extract 3.00

Yeast extract 3.00

Protease peptone 5.00

Lactose 10.00

Dextrose 1.00

Ferrous sulphate 0.20

Sodium chloride 5.00

Sodium trisulphate 0.30

Phenol red 0.024

Agar 15.00

Final pH 7.4 ±0.2(at 25 °C).

Preparation:

Suspend 57.52 grams in 1000 ml distilled water. Heat to boil to dissolve the medium completely. Mix well and distribute into tubes. Sterilize by autoclaving at 15 lbs pressure (121 ° C) for 15 minutes. Cool the tubes on slopes with inch butts.

7: Peptone Water (HiMedia Laboratories Pvt. Ltd. Mumbai, India).

Ingredients g/L

Peptic digest of animal tissue 10.00

Sodium chloride 5.00

Final pH7.2+/- 0.2(at 25°C).

Preparation:

Suspend 15.0 grams in 100 ml distilled water. Mix well and dispense into tubes with or without inverted Durham's tubes and sterilize by autoclaving at15 lbs pressure (121 °C) for 15 minutes.

8: Kovac's Reagent (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients g/L

P-dimethyl amino benzaldehyde 10 g

Isoamyl alcohol 150mL

Concentrated hydrochloric acid 50mL

Preparation:

Kovac's reagent is prepared by dissolving 10 gm of p-dimethyl amino benzaldehyde in 150 ml of isoamyl alcohol and then slowly adding 50 ml of concentrated hydrochloric acid.

9: Urea Agar (Christensen) (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients g/L

Peptic digest of animal tissue 1 .00

Dextrose 1.00 Sodium chloride 5.00

Di potassium phosphate 1.20

Mono potassium phosphate 0.80

Phenol red 0.012

Agar 15.00

Final pH 7.4±0.2 (at25°C).

Preparation:

Suspend 21 grams in 950 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 ° C) for 15 minutes. Cool to 50°C and aseptically add 50 ml of sterile 40% urea solution and mix well. Dispense into sterile tubes and allow setting on slanting position. Don't over heat or reheat the medium as urea decomposes very easily.

10: Simmons Citrate Agar (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredients g/L

Magnesium sulphate 0.20

Ammonium dihydrogen phosphate 1.00

Dipotassium phosphate 1.00

Sodium citrate 2.00

Sodium chloride 5.00

Bromothymole blue 0.08

Agar 15.00

Final pH 7.4±0.2(at25°C).

Preparation:

Suspend 24.28 grams in 100 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense as desired in tubes or flasks, sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 minutes.

11: Muller Hinton agar

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.

Ingredients g/L

Casein acid hydrolysate 17

Beef heart infusion 2

Starch soluble 1.5

Agar 17

Final pH 7.2 +/- 0.2(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 (1 21°C) for 15 min. mix and pour.

12: McFarland's standard (HiMedia Laboratories Pvt. Ltd. Mumbai, India).

Ingredient g/1

Sulphuric acid 1.0 ml

Barium chloride 2.35g

Distilled water 299 ml

Preparation:

Prepare 1% v/v solution of sulphuric acid by adding 1 ml of concentrated sulphuric acid to 99 ml of distilled water, prepare 1.175% w/v solution of barium chloride in 200 ml of distilled water, to make turbidity standard add0.5ml of barium chloride to 99.5 ml of sulphuric acid and mix.

Appendix:

Colored plate (1): Biochemical reaction of *P.mirabilis*

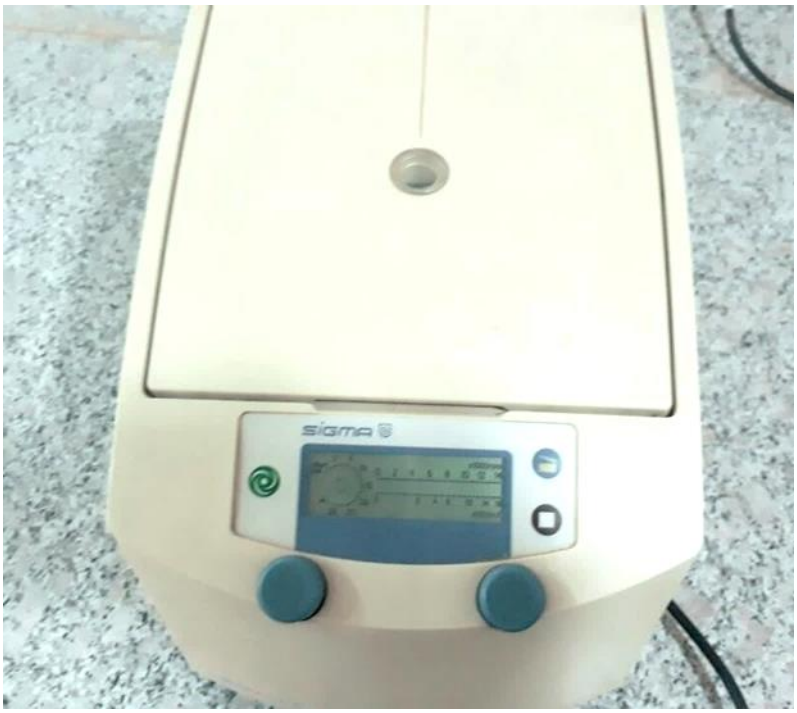
Colored plate (2): Susceptibility test results: Using ceftazidime (CAZ) 30 mcg, ciprofloxacin (CIP) 5mcg, (C) 30 mcg, gentamicin (GEN) 10 mcg and imipenem (IPM) 10 mcg on Muller Hinton agar.

Colored plate (3): Microwave

Colored plate (4): Thermocycler

Colored plate (5): Gel electrophoresis and power supply device

Colored plate (6): Transilluminator system



Color plate (7): Microcentrifuge device