بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Detection of Plasmid Mediated AmpC β-Lactamase Genes in Enterobacteriaceae Isolates from Hospitalized Patients in Khartoum State

الكشف عن جينات البيتا لاكتامز(س) التي تنقل بوساطة البلازميد في عزلات البكتيريا المعوية من المرضي بمستشفيات ولاية الخرطوم

A Dissertation Submitted in Partial Fulfillment for the Requirements of M.Sc. Medical Laboratory Science (Microbiology)

By

Anfal Ibrahim Bahereldeen Adam

(B.Sc (Honors) Medical Laboratory Science, Al Neelain University, 2017)

Supervisor

Prof. Humodi A. Saeed

Professor of Microbiology

February, 2020

الآية

بسم الله الرحمن الرحيم

قال تعالى:

وَمَا تَوْفِيقِي إِلَّا بِاللَّهِ ۚ عَلَيْهِ تَوَكَّلْتُ وَإِلَيْهِ أُنِيبُ

صدق الله العظيم

(سورة هود: الآية 88)

DEDICATION

I dedicate this work to:

My parents, husband, sons, sister, brothers, relatives, friends and teachers.

ACKNOWLEDGEMENTS

First and foremost, praise to ALLAH, who give me the strength to complete this work.

I indebted to my wonderful supervisor

Prof. Humodi A. Saeed for support and guidance.

Also thanks express to the staff of Microbiology Department for his cooperation and help.

ABSTRACT

Plasmid-mediated class C β-lactamases are reported from Enterobacteriaceae with increasing frequency. They likely originate from chromosomal AmpC of certain Gram-negative bacterial species and subsequently are mobilized onto transmissible plasmids.

The objective of this study was to detect plasmid mediated AmpC β-lactamase among Enterobacteriaceae isolates from hospitalized patients in Khartoum State, Sudan using phenotypic test and multiplex PCR Assay.

A total of 76 clinical isolates of Enterobacteriaceae were included in this descriptive-cross sectional laboratory based study. The isolates were collected from hospitalized patients in Khartoum State, including both males and females with different age using non-self-constructing information form. The isolates were stored in 20% glycerol peptone medium and inculated in MacConkey agar**.** Antbiotic susceptibitity test was carried out using Modified Kirby Bauer technique. Confirmation of the AmpC β-lactamase production was done by Cefoxitin-cloxacillin double disk synergy test. DNA was extracted using guanidine chloride method and AmpC β-lactamase genes was detected by used multiplex PCR assay.

Out of 76 isolates; 9(22.0%) isolates were found positives by Cefoxitin-cloxacillin double disk synergy test. Plasmid mediated AmpC β-actamase genes were detected only among 6(7.9%) of the total isolates.

Detection of Amp C production is crucial in order to establish the antibiotic therapy and to attain the favorable clinical outcomes.

ملخص الاطروحة

تم اكتشاف أنزيمات البيتا لاكتاماز (س) التي تنتقل بوساطة البلازميد بكثرة بين الباكتريا المعوية. من المحتمل أنها تنشأ من أنزيمات البيتا لاكتاماز (س) التي توجد علي كروموسومات بعض أنواع العصويات سالبة الجرام وبعد ذلك تنقل بواسطة البلازميدات.

الهدف من هذه الدراسة هو الكشف عن أنزيمات البيتا لاكتاماز(س) التي تنقل بوساطة البلازميد في عزلات البكتيريا المعوية من المرضي بمستشفيات ولاية الخرطوم.

شملت هذه الدراسة الوصفية ست وسبعون (76) عينة من العصويات المعوية المعزولة من عينات مختلفة من المرضي المنومون بمستشفيات ولاية الخرطوم. تم عزل العينات من الذكور والاناث بمختلف الاعمار. ومن ثم حفظها في في وسط الجليسرول بيبتون بتركيز 20 % لحين زراعتها في الماكونكي اجار. اجريت اختبارات الحساسية للمضادات الحيوية باستخدام تقنية الكاربي باور. ومن ثم اجريت فحص الحساسية التاكيدي بستخدام الكلوكلسلين سيفاتوكسين القرص المزدوج. استخلص الحمض النووي باستخدام طريقة الجوانودين كلورايد. وتم الكشف عن سلالات جينات البيتا لاكتاماز (س) بواسطة تفاعل البلمرة التسلسلي.

عزلات) من بين ست وسبعون عزلة سريرية من البكتريا المعوية التي تم اختبارها ، تم العثور على 9 (22.0٪) إيجابية عن طريق اختبار الكلوكلسلين سيفاتوكسين القرص المزدوج تم الكشف عن ست جينات فقط (% 7.9)6 من بينهم خمس من نوع ال (CIT) وواحدة من نوع ال (DHA).

يعد اكتشاف أنزيمات البيتا لاكتاماز (س) أمرًا بالغ الأهمية من أجل استخدام العلاج بالمضادات الحيوية وتحقيق النتائج السريرية المواتية.

TABLE OF CONTENTS

	Subjects	Page No.
	الاية	I
	Dedication	II
	Acknowledgements	III
	Abstract, English	IV
	Abstract, Arabic	V
	Tables of contents	VI
	List of tables	X
	List of figures	XI
	Abbreviations	XII
CHAPTER I: INTRODUCTION AND OBJECTIVES		
1.1	Introduction	1
1.2	Rationale	2

1.3	Objectives	3
1.3.1	General objective	3
1.3.2	Specific objectives	3
CHAPTER II: LITERATURE REVIEW		
2.1	History of AmpC β-lactamase	4
2.2	Mechanisms of antibiotics resistance among Gram negative rods	4
2.3	β-lactam antibiotic	4
2.3.1	Types of β-lactam antibiotics	5
2.3.1.1	Penicillins	5
2.3.1.2	Cephosporins	5
2.3.1.3	Carbapenems	6
2.3.1.4	Monobactams	6
2.3.1.5	β-lactam inhibiter	6
2.3.2	Mechanisms of action of β-lactam antibiotics	6
2.3.3	Inducing abilities of β-Lactam	7
2.3.4	Mechanisms of bacteria resistance to β-lactam antibiotics	7
2.4	β-lactamases enzymes	7
2.4.1	Classification of β-lactamase enzymes	7
2.5	AmpC β-lactamases	8
2.5.1	Types of AmpC β-lactamases	8

2.5.1.1	Chromosomal mechated AmpC β-lactamases.	8
2.5.1.2	Plasmid mediated AmpC β-lactamases	8
2.5.2	Regulation of AmpC β-Lactamase enzymes and mechanisms of resistance	9
2.5.3	Epidemiology of plasmid mediated AmpC β-lactamase infection	10
2.5.4	AmpC β-lactamase associated diseases	11
2.5.5	Detection of AmpC β-lactamases	11
2.5.5.1	Screening tests	11
2.5.5.2	Phenotypic methods	12
2.5.5.3	Genotypic detection methods	12
2.5.6	Prevention and control of AmpC β-lactamase producing bacteria	13
2.6	Previous studies	13
CHAPTER III : MATERIALS AND METHODS		
3.1.	Study design	15
3.2	Study duration	15
3.3	Study area	15
3.4	Study sample	15
3.5.	Sample size	15
3.6.	Ethical consideration	15
3.7	Laboratory methods	16
3.7.1	Collection of samples	16

3.7.2	Bacteriological methods	16
3.7.2.1	Subculture	16
3.7.2.2	Identification	16
3.7.2.2.1	Macroscopical examination	16
3.7.2.2.2	Gram stain	16
3.7.2.2.3	Biochemical tests	16
3.7.2.2.3.1	Oxidase test	16
3.7.2.2.3.2	Indole Production test	17
3.7.2.2.3.3	Citrate Utilization test	17
3.7.2.2.3.4	Urease test	17
3.7.2.2.3.5	Motility test	17
3.7.2.2.3.6	Fermentation of sugar and production of H_2S and gas	17
3.7.2.2.4	Antimicrobial susceptibility testing	18
3.7.2.2.4.1	Modified Kirby-Bauer disk diffusion method	18
3.7.2.2.4.2	Cefoxitin-cloxacillin double disk synergy test	18
3.7.3	Genotypic detection	19
3.7.3.1	Purification of bacteria	19
3.7.3.2	DNA extraction using guanidine chloride method	19
3.7.3.3	Polymerase chain reaction	19
3.7.3.3.1	Primers	19

3.7.3.3.2	Preparation of primers	19
3.7.3.3.3.	Preparation of reaction mixture for multiplex PCR	20
3.7.3.3.4	Preparation of reaction mixture for singleiplex PCR	20
3.7.3.3.5	Protocol used for amplification of the genes	20
3.7.3.3.6	Visualization of PCR products	21
3.8	Statistical analysis	21
CHAPTER IV: RESULTS		
4	Results	22
4.1.	Gender and age groups	22
4.2.	Entity of the isolates	23
4.3.	Types of clinical specimens	23
4.4	Results of susceptibility test	24
4.5	Cefoxitin-cloxacillin double disk synergy (CC-DDS) test	25
4.6	Frequency of AmpC β-lactamases genes groups among the isolates	26
4.7	Association between plasmid mediated AmpC β-lactamase genes and age groups, gender, clinical specimens and type of organisms	27
CHAPTER VI: DISCUSSION, CONCLUSION AND RECOMMENDATIONS		
5.1	Discussion	29
5.2	Conclusion	30
5.4	Recommendations	30
	References	31

	Appendix I	39
	Appendix II	40
	Appendix III	41
	Appendix IV	45

LIST OF TABLE

Table No.	Legend	Page No.
3.1	Primers specific for plasmid mediated AmpC β-lactamase genes	19
4.1	Results of susceptibility test	25
4.2	Distribution of clinical isolates screened positive for Amp C β-lactamases by Cefoxitin-cloxacillin double disk synergy (CC-DDS) test	25
4.3	Frequency of plasmid mediated AmpC β-lactamase genes among the isolates	26
4.4	Association between plasmid mediated AmpC β-lactamase genes and age groups, gender, clinical specimens and type of organisms	27

LIST OF FIGURE

Figure No.	Legend	Page No.
4.1	Distribution of isolates according to sex	22
4.2	Distribution of isolates according to age groups	22
4.3	Entity of isolates included in the study	23
4.4	Distribution of various isolates according to specimen types	24
4.5	Positive Cefoxitin-cloxacillin double disk synergy (CC-DDS) test	26
4.6	Amplified DNA of plasmid mediated AmpC β-lactamase genes. Lane 1, DNA lader 100bp; lane 3, 4, 13 and 15 are typical band size of 462pb corresponding to the molecular size of CIT gene and lane 10 was typical band size of 405pb corresponding to the molecular size of DHAgene	27

ABBRREVATIONS

ESBLs	Extended spectrum β-lactamase
MDR-GNRs	Multidrug resistant-gram negative rods
CC-DDS	Cefoxitin-cloxacillin double disk synergy test
AmpC	Class C (group 1) β–lactamace
K.pneumoniae.	*Klebsiella pneumonia*
A. baumannii	*Acintobacter baumannii*
P. aeroginosa	*Pseadomonas aeroginosa*
E. coli.	*Escherichia coli*
P. meribles	*Proteus meribles*
E. cloacae	*Enterobacter cloacae*
β-lactamase	Beta-lactamase
P. aeroginosa	*Pseudomonas aeroginosa*
S. marcescens.	*Serreatia marcescens*
C.freundii	*Citrobacter freundii*
MGEs	Mobile genetic elements(MGEs)

AMEs	Aminoglycosidases-modifyiny enzymes(AMEs)
UTI	Urinary tract infection
CIAT	Ceftazidime- imepenem disc antagonism test (CIAT)
KIA	Kligler iron agar
PBPs	Penicillin-binding-proteins
DNA	Deoxe- ribo-nuclealase
NS	Normal saline
PCR	Polymerase chain reaction
MH	Muller Hinton
NCCLS	National Committee for Clinical Laboratory Standards
mg/L	Mill gram per liter
E-test	Epsilometer test
mcg/disc	Microgram per disc
CMY	Cephamycin
FOX	Cefoxitin.
MOX	Moxalatem
LAT	Latamoxif
ACC	AMBLER CLASS C
MIR	Miriam hospital in Providence
DHA	Dharan hospital in Saudi arabia

CHAPTER I
INTRODUCTION

CHAPTER I
INTRODUCTION

1.1. Introduction

Enterobacteriaceae are major cases of community and health care acquired infection (Paterson, 2006). β-Lactamase production is the main mechanism underlying resistance to β-lactam antibiotics in Enterobacteriaceae. β-Lactamase genes are often located on plasmids or transposons, making them readily transmissible among different species (Normark *et al*., 2002). AmpC class β–lactamases are cephalosporinases that are poorly inhibited by clavulanic acid, they can be differentiated from other extended spectrum β-lactamases (ESBLs) by their ability to hydrolyse cephamycins like cefoxitin as well as other extended spectrum cephalosporins (Jacoby, 2009). Members of the family Enterobacteriaceae such as *Enterobacter spp, Citrobacter freundii* and *Serratia marcescens* have chromosomally encoded AmpC class β-lactamases, plasmid-mediated AmpC β-lactamases derived from the chromosomal encoded AmpC β-lactamases and reported most in *Klebsiella spp, Salmonella spp, Enterobacter aerogenes*, and *Proteus mirabilis* (Coudron *et al*., 2000 and Philippon, 2002). During the last decade plasmid-mediated AmpC-type β-lactamases have been increasingly isolated worldwide and linked to treatment failure. (Jacoby, 2009).

Detection of AmpC-producing Enterobacteriaceae is significant clinical relevance, since AmpC producers may seem susceptible to expanded-spectrum cephalosporin when initially tested, this may lead to inappropriate antimicrobial regimens and therapeutic failure (Pai *et al*., 2004). Carriage of plasmid mediated AmpC is often associated with multidrug resistance (e.g. resistance to aminoglycosides, quinolones and co-trimoxazole) and bacteria producing AmpC β-lactamase enzyme are more likely to become resistant to carbapenems (Pitout, 2012, Rodriguez-Bano *et al*., 2012). A number of detection methods for Bla-AmpC were proposed, the comparative sensitivity

and specificity of these methods has not been comprehensively studied. The lack of an agreed AmpC β-lactamase detection methods hinders the investigation of their epidemiology and understanding of their clinical significance. Multiplex PCR for AmpC- β-lactamase genes detection is available as a research tool, but is expensive and is not yet available for routine use (Pérez-Pérez *et al*., 2002).

1.2.Rationale

The epidemiological profile of Sudan, as low and middle-income country, where malnutrition and infectious diseases are the main causes of morbidity and mortality, infections due to Enterobacteriaceae are on rise world over, the wide use of broad-spectrum antibiotics can lead to colonization with resistant strains which can transmit among people and animals and from one country to another without notice, multidrug resistant-Gram negative rods (MDR-GNRs) are most often documented in acute care facilities (Faiyaz *et al*., 2019). AmpC β-lactamase are one of the common class of β-lactamase detected from cephalosporin's resistant, it frequently assosited with production of multidrug resistance bacteria, most clinical laboratories and physicians remain unaware of their clinical importance and there are no recommendations available from the NCCLS or elsewhere for detection of organisms producing plasmid-mediated AmpC β-lactamase genes, current detection methods for organisms producing plasmid-mediated AmpC β-lactamases are technically demanding for clinical laboratories to perform on a routine basis , as a result, organisms producing these types of β-lactamase often go undetected and therefore have been responsible for several nosocomial outbreaks (Thomson, 2001). The detection of organisms producing these β-lactamase is very important for enhanced infection control and to ensure effective therapeutic options, multiplex PCR is available as a research tool for detection of plasmid-mediated AmpC β-lactamases but is not yet available for routine use in clinical laboratories (Pérez-Pérez *et al*., 2002).

There is dearth of information on documentation of plasmid-mediated AmpC β-lactamases genes in Sudan so detection of plasmid-mediated AmpC β-lactamases genes among Enterobacteriaceaeis isolates from different clinical specimens will be a great value and have important role in understanding the spread of this pathogen.

1.3. Objectives

1.3.1. General objective

To detect plasmid mediated AmpC β-lactamase genes among Enterobacteriaceae isolates from hospitalized patients in Khartoum State.

1.3.2. Specific objectives

1. To identify members of Enterobacteriaceae isolates that collected from different hospitals using convential biochemical tests.
2. To screening for cephalosporin-resistant Enterobacteriaceae using Kirby-Bauer disk diffusion method.
3. To confirm the AmpC β-lactamase production using Cefoxitin-cloxacillin double disk synergy test .
4. To detect AmpC β-lactamase genes among cephalosporin-resistant Enterobacteriaceae using multiplex PCR assay.

CHAPTER II

LITERATURE REVIEW

CHAPTER II

LITERATURE REVIEW

2.1. History of AmpC β-lactamase

Most antibiotic classes we used as medicines today such as penicillin are discovered and introduced at period between (1940-1962) which known as golden period of antibiotics (Singh *et al*., 2006). The most common mechanism for resistant to β-lactam antibiotic among Gram negative bacteria is the production of β-lactamase. In 1980 new β-lactam drugs such as cephalosporins, carbapenems and monobactams with greater β-lactamase stability introduced to meet the challenge of resistance, but the emergency of resistance to expanded spectrum cephalosporins has been a major concern (Abraham *et al*., 1940).

Initially the resistance appeared in organisms such as *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Citrobacter freundii* and *Serreatia marcescens* due to mutation in chromosomal genes result in over expression of β-lactamase resistance genes such as class C (group 1) β-lactamase (AmpC) that provide resistance to cephalosporin later on cephalosporin resistances appeared among bacterial species that lack an inducible chromosomal mediated AmpC gene such as; *Escherichia coli*, *Salmonella* spp and *Proteus meribles* , this type of resistance was mediated by plasmids encoding β-lactamase such as ESBLS genes and AmpC genes (Jacoby, 2009), in 1989, Bauernfeind *et al* described a *Klebsiella pneumoniae* isolate from South Korea that could transfer resistance to cefoxitin and cefotetan as well as to penicillins, oxyimino-cephalosporins, and monobactams to *Escherichia coli*, the first proof that a class C β-lactamase had been captured on a plasmid was provided by Papanicolaou *et al*., who described transmissible resistance to alpha-methoxy- and oxyimino-beta-lactams mediated by an enzyme (MIR-1) with

the biochemical properties of a class 1 β-lactamase and showed that part of the MIR-1 gene was 90% identical to the AmpC gene of *Escherichia cloacae*, subsequently, plasmid-mediated class C β-lactamases has been discovered worldwide (Papanicolaou *et al*, 1990).

2.2. Mechanisms of antibiotics resistance among Gram-negative rods

Intrinsic resistance due to mutation in chromosomal genes resulting in over expression of antibiotic inactivating enzymes or efflux pumps and permeability alterations by loss of outer membrane porins, or target modification (Ruppe *et al.,* 2015).

Acquired resistance by horizontal transfers of mobile genetic elements (MGEs) caring resistance genes most commonly plasmid encoding β-lactamases, aminoglycosidases-modifyiny enzymes (AMEs) and non enzymatic mechanisms such as Qnr for fluoroquinolone resistance in *Enterobacteriaceae* (Ruppe *et al*., 2015).

2.3.β-lactam antibiotics

β-lactam antibiotic are one of the widely used antibacterial agents until now, it is contain β-lactam ring (3 carbon and 1 nitrogen ring) in their molecular structure, this range act as potent inhibitors of cell well synthesis (Chast, 2008).

2.3.1. Types of β-lactam antibiotics

2.3.1.1. Penicillins

Are one of the oldest class of β-lactam antibiotic contain a nucleus of 6 aminopencillanic acid (lactam plus thiazolidine) rang and other ring side chains, penicillin derived originally from *Penicillium* moulds (Chast, 2008).

2.3.1.2. Cephosporins

Are β-lactam antibiotic original delivered from fungi *Acremonium* also known as cephalosporium they contain a 7- amino cephalosporunic acid nucleus and side chain contain 3,6 dihydro- 2h-1, 3- thiazani rings. There is 5 generation of cephalosporins grouped by their antimicrobial properties , each newer generation has significantly Gram negative antimicrobial properties than preceding generation and in most decreased activity against Gram positive bacteria (Chast, 2008).

2.3.1.3. Carbapenems

Are class of highly effective β-lactam antibiotic their structure is carbapenems coupled to beta-lactam rang that confers production against most β-lactamase and used to treat severe infections and infection caused by multi drug resistant Gram negative bacteria. Resistance to these antibiotics classes are serious issue and occur mainly among Gram negative pathogens such as *Klebsiella pneumoniae, Acintobacter baumannii* and *Pseadomonas aeroginosa* (Inverarity, 2010).

2.3.1.4. Monobactams

Are β-lcactam ring stands alone and fused to another ring and it effective only against Gram negative bacteria such as *Neisseria* and *psudomomas*. Example of monobactams; Aztreonam (Sykes, 1981).

2.3.1.5. β -lactam inhibiter

Are work primary by in activating the serine β-lactamases such as; Clavulanic acid, sulbactam and vaborbactam that active against carbapenemases such as *Klebsiella pneumonias* carbapenemases (KPC) (Elliott, 2017).

2.3.2. Mechanisms of action of β -lactam antibiotic

β-lactam inhibit the last step in peptidoglycan synthesis by acetylating the transpeptidases involved in cross-linking peptide to form peptidoglycan, the transpeptidation reaction are

important feature of bacterial cell wall synthesis and results in the cross linking of two glycan – linked peptide chains of peptidoglycan (the man constituent of bacterial cell walls) (Suarez *et al.*, 2009). The trans-peptidase enzyme that binding the penicillin or other β-lactam antibiotic are known as Penicillin binding proteins (PBPs) , when bind to penicillin are forming penicillin – PBP complex that fail to catalyze transpeptidation reaction but cell wall continues to be formed and the newly synthesized cell wall is no longer peptide bridged ,cross linked and therefore the synthesis of complete fully cross-linker peptidoglycan is blocked resulting in death of bacterial cells by osmotic lysis process. Penicillin-PBP-complex are also stimulates the release of autolytic enzymes that digest the existing cell wall and some special proteins called bacterial alanine which form holes on plasma membrane (Winn, 2006).

2.3.3. Inducing abilities of β-Lactam

Strong inducers and good substrates for AmpC beta-lactamase; benzylpenicillin, ampicillin, amoxicillin, and cephalosporins such as cefazolin and cephalothin. Inducers but are much more stable for hydrolysis; cefoxitin and imipenem. Weak inducers and weak substrates but can be hydrolyzed if enough enzyme is made; Cefotaxime, ceftriaxone, ceftazidime, cefepime, cefuroxime, piperacillin, and aztreonam (Neu, 1985).

2.3.4. Mechanisms of bacteria resistance to β-lactam antibiotic

Bacteria resistance to β-lactam antibiotic by; production of β-lactamases enzyme, decrease penetration to target site example; resistance of *Pseadomonas aeroginosa*, alteration of target site PBPs example; penicillin resistance in *Pneumococci* and efflux from the periplasmic spaces through specific pumping mechanism (Spratt, 1994, Bush *et al*., 1995 and Denyer *et al*., 2002).

2.4. β-lactamases enzymes

Are common mechanism's of resistance to β-lactam among Gram negative bacteria. β-lactamase are able to hydrolysis the chemical compounds containing in β-lactam ring C (Bush *et al*., 1995).

2.4.1. Classification of β-lactamase enzymes

The enzymes are divided into four classes based on the primary sequence similarity and catalytic mechanism. Classes A, C and D utilize an active-site serine in their mechanism of action, whereas class B β-lactamases require divalent metal cations (Zn) to catalyze β-lactam hydrolysis (Ambler, 1980). Some infectious bacteria have not only acquired β-lactamases to counteract the effects of traditional β-lactam antibiotics, but have also acquired variant forms of these enzymes to hydrolyze newly developed extended-spectrum β-lactam antibiotics (Petrosino *et al*., 1998). The new classification by Bush-Jacoby- Medeiros systems classifies the enzymes according to their substrate profiles and susceptibility to β -lactamase inhibitor such as clavulanic acid into several groups (Bush, 1989).

2.5. AmpC β-lactamases

2.5.1. Types of AmpC β-lactamases

2.5.1.1. Chromosomal mechated AmpC β-lactamases.

Some Gram negative organisms such as *Citrobacter* , *Serratia* , *Pseudomonas* or *Protous*, *Acinetobacter* and *Enterobacter* are in state of chromosomally mediated AmpC hyper production by mutation in the suitable environment, the development of resistance upon therapy is serious clinical problem. Ampicillin, amoxycillin, and cefazolin are regarded as strong inducers, excellent substrate of AmpC β-lactamases and β-lactamase inhibitors are also inducers of AmpC and can gives to treatment failure with drugs appeared susceptible invitro (Jaurin, *et al*., 1981 and Knott-Hunziker *et al*.,1982).

2.5.1.2. Plasmid mediated AmpC β-lactamases

Since, 1989 the global distribution of Gram negative bacterial strains with plasmid–encoded AmpC genes were detected among nosocomial and Enterobacteriaceae isolates. There are minor differences in amino acid sequence given rise to families of plasmid-encoded AmpC genes (Ogata *et al*., 2005), this plasmid mediated AmpC β-lactamase have been named according to the; Resistance produced by which antibiotic into (CMY family (43 alleles); Cephamycin, FOX family (7 varietes); Cefoxitin, MOX family (3 varieties); Moxalatem, LAT family (4 varieties); Latamoxif), type of β lactamase (ACC family(4 varieties)), region of discovery (MIR family (4 varieties); (MIRIAM HOSPITAL IN PROVIDENCE), DHA family (2 varieties); Dharan hospital in Saudi arabia). This plasmid enzyme-related and very closely to chromosomally determined AmpC enzymes, Examples; CMY has 6 varieties; CMY-1, 8, 9, 10,11 and 19 related

to chromosomally determined AmpC enzymes in *Aeromonas spp*. Other enzymes are related to *Citrobacter freundii*. LAT-2 was identical to CMY-2, LAT-3 was identical to CMY-6, and LAT4 was to LAT-1. The genes for ACT-1,DHA-1,DHA-2,& CMY-13 are linked to ampR genes, and are inducible. while others are not. AmpC plasmids lack ampD genes, but the expression of ACT-1 increased with the loss of chromosomal Amp D function (Higgins *et al*., 2001). Plasmids carrying genes for AmpC β-lactamases carry other gene resistance including aminoglycosides, chloramphenicol, quinolones, sulphonamides, tetracyclines, trimethoprims and TEM-1, CTX-M3,SHV varieties. The AmpC gene is usually a part of integran and the same gene can be incorporated in to different backbone on different plasmids. A variety of genetic elements are implicated in the mobilization of AmpC genes onto plasmids (Stock *et al*., 2003).

2.5.2. Regulation of AmpC β-Lactamase enzymes and mechanisms of resistance

In many Gram negative bacteria, AmpC expression is low but inducible in response to β-lactam exposure. The disruption of murein biosynthesis by β-lactam agent leads to an accumulation of N-acetylglucosamine-1,6-anhydro-N-acetylmuramic acid oligopeptides, the N-acetyl glucosamine moiety is removed to produce a series of 1, 6-anhydro-N-acetylmuramic acid tri-, tetra, and pent peptides, these oligopeptides compete with oligopeptides of UDP-N-acetylmuramic acid for a binding site on AmpR, a member of the LysR transcriptional regulator family. Displacement of the UDP-N-acetylmuramic acid peptides signals a conformational change in AmpR, which activates the transcription of AmpC (Hanson, *et al*., 2008). The cell has an enzyme, AmpD, a cytoplasmic N-acetyl-muramyl-L-alanine amidase, that removes stem peptides from the 1,6-anhydro-Nacetylmuramic acid and N-acetylglucosamine-1,6-anhydro-Nacetylmuramic acid oligopeptide derivatives, thus reducing their concentrations and preventing the over expression of AmpC, the most common causes of AmpC over expression in clinical isolates are mutation in; ampD, AmpR and AmpG which is an inner membrane permease that transports the oligopeptides involved in cell wall recycling and AmpC regulation into the cytosol (Hanson, *et al*., 2008).

Different organisms add additional features to AmpC regulation for example: *E. coli* lacks an ampR gene, consequently, AmpC in *E. coli* is non inducible but is regulated by promoter and attenuator mechanisms (Jaurin *et al*., 1981), as is AmpC production in *Shigella* (Bergstrom *et al*., 1982), *Acinetobacter baumannii* also lacks an ampR gene so that its AmpC β-lactamase is non

inducible (Bou and Martinez-Beltran, 2000), AmpC in *Serratia marcescens* is regulated by ampR, but the AmpC transcript has an unusual untranslated region of 126 bases forming a stem-loop structure that influences the transcript half-life (Mahlen *et al*., 2003), *P. aeruginosa* PAO1 has three ampD genes, explaining the stepwise up-regulation of AmpC production seen in this organism with the successive inactivation of each ampD gene (Juan *et al*., 2006). The multiple ampD loci contribute to virulence since *a P. aeruginosa* strain partially derepressed by the inactivation of one ampD allele remains fully virulent, while double or triple ampD mutants lose the ability to compete in a mouse model of systemic infection (Moya *et al*., 2008). Other aspects of AmpC regulation in *P. aeruginosa* are also more complex than that in the *Enterobacteriaceae.* AmpR is involved in the regulation of other genes besides AmpC (Kong *et al*., 2005.), an ampE gene encoding a cytoplasmic membrane protein acting as a sensory transducer has a role in AmpC expression as part of an ampD operon (Juan *et al*., 2005), and the CreBCD system as well as dacB, encoding a nonessential penicillin binding protein, are involved in AmpC hyperproduction as well (Moya *et al*., 2008). The AmpC enzyme in *Aeromonas spp*. is controlled, along with two other chromosomally encoded β-lactamases, not by an AmpR-type system but by a two-component regulator, termed brlAB in *Aeromonas hydrophila* (Alksne and Rasmussen, 1997.). BrlB is a histidine sensor kinase, the regulated β-lactamase genes are preceded by a short sequence tag (TTCAC), and an inner membrane protein is also involved in regulation, but the chemical signal for induction is not yet known (Avison *et al*., 2004).

The concentration of β-lactam substrate in the periplasm is a function of the permeability of the cell's outer membrane, in particular the presence of porin channels through which β-lactams penetrate and of efflux pumps, which transport them out of the cell. At one time, the binding of substrate to AmpC β-lactamase was entertained as a mechanism to explain resistance to β-lactam that appeared to be poorly hydrolyzed (Then and Angehrn, 1982). Decreasing the number of porin entry channels or increasing efflux pump expression can lower influx and further augment enzyme efficiency. Thus, carbapenem resistance in clinical isolates of *P. aeruginosa* involves various combinations of overproduction of AmpC β-lactamase, decreased production of the OprD porin channel for imipenem entry, and activation of MexAB-OprM and other efflux systems (Quale *et al*., 2006). Also, cephalosporins with both positive and negative charges (i.e; zwitterionic molecules) such as cefepime and cefpirome have the advantage of penetrating the outer bacterial membrane more rapidly than those with a net positive charge, such as cefotaxime

and ceftriaxone, thus more easily reaching their lethal targets without -lactamase inactivation (Nikaido and Rosenberg, 1990).

2.5.3. Epidemiology of plasmid mediated AmpC β-lactamase infection

Plasmid-mediated AmpC β-lactamases are worldwide distributed but it is less common than extended-spectrum β-lactamases (ESBLs), the lactamase CMY-2 has the broadest geographic spread and is an important cause of β-lactam resistance in nontyphoid *Salmonella* strains in many countries, CMY-2 has been responsible for ceftriaxone resistance in *Shigella sonnei* outbreak, most other strains with plasmid-mediated AmpC enzymes have been isolated from patients after several days of hospitalization, but recently, AmpC-producing isolates in cultures from long-term care facilities, rehabilitation centers, and outpatient clinics have been reported (Migliavacca *et al*., 2007).

Risk factors for bloodstream infections caused by AmpC-producing strains of *K. pneumoniae* include long hospital stay, care in an intensive care unit (ICU), central venous catheterization, need for an indwelling urinary catheter, and prior administration of antibiotics, especially broad-spectrum cephalosporins and β-lactamase inhibitor combinations, and are thus similar to risk factors for infection by ESBL-producing *K. pneumoniae* strains (Pai *et al*., 2004).

2.5.4. AmpC β-lactamase-associated diseases

AmpC producing bacteria play a major role in health care facilities as the pathogen that causes Nosocomial infection, hospital acquired infection, wound infection, urinary tract infection, VAP, meningitis, septicemia, CAUTI and risk of infection via food with the different type of pathogens , especially *Salmonella*, *EHEC* , *Klebsiella* (Chow *et al*., 1991, Yan *et al*., 2004). Mortality and morbidity increased in immunocoprmised patients, than immune competent. (Pai *et al*., 2004). The harmless intestinal bacteria can pass on the genes for Amp C to pathogenic bacteria, such as ‘ *Salmonella*’ Infections are transmitted by food stock home handlers, pet animal handlers, health care workers, so the risk of infections occurred by in between animals or animals to human by pet handlers (Yan *et al*., 2004)

2.5.5. Detection of Amp C β-lactamase

There are presently no CLSI or other approved criteria for AmpC detection (Doi and Paterson, 2007), organisms producing enough AmpC-lactamase will typically give a positive ESBL screening test but fail the confirmatory test involving increased sensitivity with clavulanic acid (Bell *et al*., 2007)

2.5.5.1. Screening test

Kirby-Bauer disk diffusion method and dilution methods to determine the MICs of cefoxitin (CLSI, 2015).

2.5.5.2. Phenotypic methods

Boronic acid inhibitor based test; Boronic acids have long been known as AmpC inhibitors . Various boronic acid derivatives have been either added to a blank disk placed near a β-lactam disk or added to the β -lactam disk for comparison with an unmodified β-lactam disk (Yagi *et al.*, 2005), AmpC disc test: these test is based on use of Tris-EDTA to permeabilize a bacterial cell and release β-lactamases into the external environment, disc antagonism test; Simple and cost-effective first-line tests for AmpC-type -lactamase determination, indicating the presence of an AmpC-type -lactamase, and further providing a specific means of detection of the ACC-1-lactamase, ceftazidime-imepenem disc antagonism test (CIAT); it a phenotypic test to detect and confirm the presence of inducible AmpC β-lactamases based on the strong inducing effect of imipenem on these enzymes (Livermore and Brown, 2001), cefoxitin-cloxacillin double disk synergy test (CC-DDS); This test will be conducted based on the inhibitory effect of cloxacillin on AmpC production, modified three dimension test; was designed to detect both AmpC and ESBL production. In the indirec form used for AmpC detection (Tan *et al*., 2009), three-dimensional enzyme extraction assay; this assay was performed as described by (Coudron *et al*., 2000). In view of the fact that carbapenemases may hydrolyse cefoxitin, the presence of enhanced growth of organisms at the point on the agar surface where the slit intersected the zone of inhibition was only taken as evidence of Bla-AmpC when in combination with negative carbapenemases testing (Shahid *et al*., 2004). E-test strips with a gradient of cefotetan or

cefoxitin on one half and the same combined with a constant concentration of cloxacillin on the other half have been evaluated for AmpC detection (Bolmstrom *et al*., 2006).

2.5.5.3. Genotypic detection methods

Phenotypic test cannot distinguish various families of plasmid mediated Amp C enzymes but, also identified chromosomally determined AmpC enzymes with an ESBL, for this, the current method of gold standard for plasmid-mediated AmpC detection, Multiplex PCR has been improved by utilizing Six primer pairs, to which a seventh pair for CEF-1 β-lactamase could be added, Compared to ESBL producers, isolates producing AmpC β-lactamase are resistant to additional β-lactams and β-lactam inhibitors and developing resistance to Carbapenems (Knox *et al*., 1996).

2.5.6. Prevention and control of AmpC β-lactamase producing bacteria

Common principle of control measures are hand washing standard precaution and scrupulous hand hygiene are used. Area of concern, particularly transmission of infection occur in neonatal ward surgical ward, ophthalmic ward, post operative ward, & important of burns patient care are needs intervention therapy. There are different type of multi-drugs resistance Gram negative rods (MDR-GNB) are greater or lesser concern, flexibly judged by Microbiologist, infection prevention and control team, so continuous screening, review and diagnosis are done, drug resistant pattern are continuously monitored, decontamination procedures are followed, excessive usage of antimicrobials are avoided or controlled by Health board team and avoid reusable drugs infection surveillance is very important (George, 2009).

2.6. Previous studies

In the United States, DHA, ACT-1/MIR-1, and FOX-type enzymes were the most common genes reported by (Song, *et al*., 2006) and in Japan, MOX-1, CMY-9,CMY-19, CMY-1, CMY-2 and DHA-1 were the most common genes found in clinical isolates as reported by (Moland *et al*., 2006).

In a study by Soha A, *et al*., 2008*;* both CMY-1 and CMY-2 were the most common genes detected in their region at Egypt.

In Korea study by (Li Y, *et al*., 2008) found that DHA, CMY/MOX, and ACT-1/MIR-1type enzymes were predominant.

Wassef, *et al*., 2014; reported that 22 AmpC genes were detected in 25.8% of the positive cefoxitin screened isolates of which 40.9% belonged to each of the MOX and the FOX families, 13.6% belonged to the EBC family, and 4.5% belonged to the CIT family.

Some study demonstrate synergy arising from inhibition of ESBL by Clavulanate in the presence of AmpC (Anitha *et al*., 2016).

In Saudi Arabia; Mutasim et al., 2019 detected that out of three hundred eleven GNB were collected from clinical specimens of patients; 32.5%(101) were AmpC β-lactamase producers and AmpC β-lactamases were found more frequently among *Acinetobacter spp, P.aeruginosa* and *Enterobactercloacae.*

In Khartoum State, Sudan, only investigation that conducted by Maha *et al*., 2020, AmpC beta-lactamases were screened using AmpC disk test , out of total 168 Enterobacteriaceae recovered from clinical samples of patients during May 2014 to February 2015; AmpC beta-lactamase detected as 49.3%, with peak levels among *Acinetobacter baumannii* (83.3%) and *Enterobacter cloacae* (75%).

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1. Study design

This was a laboratory-based descriptive-Cross sectional study.

3.2. Study duration

The study was conducted during period from March 2019 to February 2020

3.3. Study area

Enterobacteriaceae isolates were collected from Royal Care International Hospital, East Nile Hospital and AL Moalem Medical City. The study was performed at Sudan University of Science and Technology, College of Graduated Studies.

3.4. Study sample

The Eenterobacteriaceae isolates were obtained from a variety of clinical specimens (e.g. urine, pus, sputum, blood, body fluid, tip of central line, eye swab, wound swab and tissues) isolated from different hospitalized patients.

3.5.Sample size

The total of seventy six isolates (n= 76) belonging to the family Eenterobacteriaceae were collected.

3.6. Sampling techniques

Non-probability convenience sampling technique

3.6. Ethical consideration

Ethical approval was obtained from Research Ethics Committee of Sudan University of Science & Technology and verbal consent from laboratory administration has been also provided and approved the study.

3.7. Laboratory Methods

3.7.1. Collection of isolates

The isolates were collected from microbiology laboratory from targeting hospitals in screw-capped tubes containing 5ml of 20% peptone glycerol broth and preserved at 4ºC refrigerator at Research Laboratory in Sudan University of Since and Technology. Then subculture for re identification and DNA extraction.

3.7.2. Bacteriological methods

3.7.2.1. Subculture

Under aseptic conditions from 20% peptone glycerol broth stock the bacteria were sub-cultured into MacConkey and incubated at 37˚C for 24 hours. The growth on MacConkey agar was sub-cultured on the slope of Nutrient agar by using sterile straight wire in zigzag form for further tests.

3.7.2.2. Identification

3.7.2.2.1. Macroscopical examination

The comment on colonial morphology on MacConkey agar growth was performed with regard to the size, appearance, color, edge, side view, smell and pigment production of the colonies.

3.7.2.2.2. Gram stain

The Gram's was done by preparing smear from MacConkey agar colony, by taking loop full of normal saline by sterile loop in slide and just touch from the colony and emulsified in the normal saline to make smear then let smear to dry and fixed by heating, then mear was covered with crystal violet for (30-60 seconds) followed by lugol‘s for (30-60seconds) then decolorizer

(alcohol) was added for few seconds and finally the counter stain (safranine) for(30-60seconds) smear was leaved to air dry and examined by oil immersion lens. G-gram negative bacilli were stained red (Cheesbrough, 2000).

3.7.2.2.3. Biochemical tests

3.7.2.2.3.1. Oxidase test

The test organisms was rubbed over the reagent impregnated, filter paper disc (Oxidase disc) using sterile applicator sticks. Controls were also kept along with the test and the reaction was observed within 10 seconds. Formation of purple color indicates a positive test. No color changes show a negative test (Cheesbrough, 2000).

3.7.2.2.3.2. Indole production test

The peptone water tubes were inoculated with bacterial colonies using sterile striate loop, an uninoculated tube was kept as control negative, both tubes were incubated at 37°C for 24-48 hours, after proper incubation, 1 ml of Kovac's reagent was added to both tubes including the control then the tubes were shaken gently after an interval for 10 – 15 minutes the tubes were observed for the color in the top reagent layer; negative when the layer color is yellow and positive when the layer color are pink (Cheesbrough, 2000).

3.7.2.2.3.3. Citrate utilization test

By used sterile technique Simmons citrate agar slant was inoculated with the test organism by means of a stab and streak inoculation, an uninoculated tube was kept as control and both tubes were incubated at 37°C for 24 – 48 hours then observed the tubes for growth and coloration of the medium. Interpretation according to the color of the medium; a positive result is indicated when color of media turned to blue color and if color of the medium remains as green, indicates a negative result (Cheesbrough, 2000).

3.7.2.2.3.4. Urease test

By used sterile technique, the test organism was inoculated the Christener's urea agar slant media by means of loop of inoculation, an uninoculated tube was kept as control, the tubes were incubated at 37°C for 24-24 hours and the reaction was observed to see if pink color has developed or not, development of pink color indicated a positive test and no color change indicated a negative test (Cheesbrough, 2000).

3.7.2.2.3.5. Motility test

By used sterile technique the test organism was inoculated in to semi solid medium used stab inoculation method, an uninoculated tube was kept as control, both tubes were incubated at 37°C for 24-48 hours and the reaction was observed for motility as; Diffused growth – Motile bacteria and growth at stab line only – Non-motile bacteria (Cheesbrough, 2000).

3.7.2.2.3.6. Fermentation of sugar and production of H_2S and gas

Under aseptic condition, by using sterile straight wire, the Kligler Iron agar was inoculated with testing organism , first the butt was stabbed then the slope was streaked , and incubation was done at 37˚c for overnight, the reaction was observed for the color of both the butt and slant and gas production by means of cracks or bubble and H2S production by blackness of butt (Cheesbrough, 2000).

3.7.2.2.4. Antimicrobial susceptibility testing

3.7.2.2.4.1. Modified Kirby-Bauer disk diffusion method

By used a sterile wire loop 3–5 well-isolated colonies were taken and emulsified in 3–4 ml of sterile physiological saline and the turbidity of the suspension was matched with the turbidity of Mcfarland standard then by used a sterile cotton swab, a plate of Mueller Hinton agar(MHA) was inculated and excess fluid was removed by pressing and rotating the swab against the side of the tube above the level of the suspension, swab was streaked evenly over the surface of the medium in three directions, the plate was rotated approximately 60^o to ensure even distribution, then the antibiotic discs from different categories including: extended spectrum cephalosporins (ceftazidime (30 µg)), Cephamycin (cefoxitin (30 µg)), carbapenems (imipenem (10 µg)), aminoglycosides (amikacin (30 µg)), fluoroquinolones (ciprofloxacin (5 µg)), phenicol (chloramphenicol (30 µg)) and polymyxin (colistin (10 µg)) (Oxoid, UK) were placed on the surface of Mueller Hinton agar(MHA) gently by using sterile forceps (Cheesbrough, 2000), *E. coli* ATCC 25922 used as control strains and tested each time when susceptibility testing performed, then the plates were incubated overnight at 37^oC. Interpretation of results was done by used a ruler on the underside of the plate, the diameter of each zone of inhibition were measured in mm and by using the Interpretative Chart, the zones sizes of antibiotic disks were interpreted and was reported the organism as; Resistant ,intermediate or sensitive , as per CLSI recommendations (CLSI, 2015) (Cheesbrough, 2000).

3.7.2.2.4.2. Cefoxitin-cloxacillin double disk synergy test (CC-DDS)

Cefoxitin disks (30 µg) was used for screening of AmpC-producing isolates, isolates exhibiting inhibition zones with diameter ≤ 18 mm considered positive for AmpC screening and will be subjected to further testing using Cefoxitin-cloxacillin double disk synergy test (CC-DDS), this test was conducted based on the inhibitory effect of cloxacillin on AmpC production, 30µg cefoxitin disks (Oxoid, UK) supplemented with 200 µg cloxacillin was used, the isolates (comparable to 0.5 McFarland standards) were inoculated on Mueller Hinton agar, then the diameters of the cefoxitin inhibition zones were compared with and without cloxacillin, an increase in zone size of ≥ 4 mm for cefoxitin with and without cloxacillin indicated AmpC production as described by (Tan *et al*., 2009)

3.7.3. Genotypic detection

3.7.3.1. Purification of bacteria

All tested isolates were purified by subcultering the single well identified colony from MacConkey agar into nutrient ager media and incubated over night at 37^{o}C.

3.7.3.2. DNA extraction using guanidine chloride method

DNA extracted by used guanidine chloride method, all scraped colonies were washed with normal saline (NS), flowed by addition of 400µL lysis buffer, 5µL proteinase K, 200µL guanidine chloride and 50µL ammonium acetate, suspension were inculated overnight at 37^{o}C; on the next day 400µL of cooled chloroform were added and the mixture were centrifugated for 5min at 21RPM then the 400µL from clear upper layer were collected in new tube and 1mL of cold absolute ethanol was added, mixed and freeze overnight. Next day the pellet was taken from freezer and kept at room temprterure for 5min than centrifugated for 5min than the 100% ethanol was discarded and the pellet was washed by cold 70% ethanol and allowed to dry, then the pellet was suspended with 120µL nuclease-free water and qualified using gel electrophoresisand the DNA samples were stored at -20 ^{o}C until used for PCR (Sabeel *et al*., 2017).

3.7.3.3. Multiplex polymerase chain reaction (PCR)

3.7.3.3.1. Primers

Published primer pairs for AmpC β-lactamase antimicrobial resistance genes were ordered from Macrogen Company, Korea **(Table 3.1)** The primers were dissolved according to manufacture guide lines to prepare 100pmol/µl.

Table 3.1 Primers specific for plasmid mediated AmpC β-lactamase genes

Family	Primer Sequence (5' to 3')		Amplicon Size (bp)	References
MOX	F	GCT GCT CAA GGA GCA CAG GAT	520	(Pérez-Pérez and Hanson, 2002)
	R	CAC ATT GAC ATA GGT GTG GTG C		
CIT	F	TGG CCA GAA CTG ACA GGC AAA	462	
	R	TTT CTC CTG AAC GTG GCT GGC		
DHA	F	AAC TTT CAC AGG TGT GCT GGG T	405	
	R	CCG TAC GCA TAC TGG CTT TGC		
EBC	F	TCG GTA AAG CCG ATG TTG CGG	302	
	R	CTT CCA CTG CGG CTG CCA GTT		
FOX	F	AAC ATG GGG TAT CAG GGA GAT G	190	
	R	CAA AGC GCG TAA CCG GAT TGG		
ACC	F	AAC AGC CTC AGC AGC CGG TTA	346	
	R	TTC GCC GCA ATC ATC CCT AGC		

3.7.3.3.2. Master Mix

Premix Kit (iNtRON BIOTECHNOLOGY, Korea); which is a maxima PCR ready to use premixed solution containing all reagents required for PCR (except water, template and primers), and additional compound needed for direct loading onto agarose gel and tracking dye [blue] that allow monitor progress during the electrophoresis, were used.

3.7.3.3.3. Preparation of reaction mixture for multiplex PCR

Total reaction volume was 25 µl in 0.2 ml eppendorff tube; 5 µl deionized sterile water was added to; 5 µl Maxima PCR Premix Kit (iNtRON BIOTECHNOLOGY, Korea), 0.5 µl from each of the sex forward primer (Macrogen, Korea), 0.5 µl from each of the sex reverse primer (Macrogen, Korea) and 3 µl plasmid DNA (template DNA).

3.7.3.3.4. Preparation of reaction mixture for singleiplex PCR

Total reaction volume was 25 µl in 0.2 ml eppendorff tube contain 5 µl deionized sterile water, 5 µl Maxima PCR Premix Kit (iNtRON BIOTECHNOLOGY, Korea), 0.5 µl forward primer (Macrogen, Korea), 0.5 reverse primer (Macrogen, Korea) and 2µl plasmid DNA (template DNA).

3.7.3.3.5. Protocol used for amplification of the genes

The amplification was done using thermal cycler (Convergys, Germany), the PCR mixture was subjected to initial denaturation step at 94°C for 3min, followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 64°C for 30 seconds, followed by step of elongation at 72°C for 60 seconds and the final elongation was carried out at 72°C for 7min.

3.7.3.3.6. Visualization of PCR products

The amplicons were separated at 50V for one hours in a 1.5% agarose gel containing ethidium bromide, the bands were visualized by U.V transilluminater (Uvite –UK) to detect the specific amplified products by compared with 100bp standard DNA ladder (marker)(INtRON biotechnology Korea) injected for each run.

3.8. Statistical analysis

Data obtained were analyzed and presented using Statistical Package for Social Sciences (SPSS; Version16.0). Categorical variables were compared with the chi-square test. Statistically significant was set at A p-value of <0.05.

CHAPTER IV
RESULTS

CHAPTER IV
RESULTS

4. Results

4.1. Gender and age groups

A total of seventy-six Enterobacteriaceae isolates from hospitalized patients were included in this study to detect the presence of plasmid mediated AmpC β-lactamase genes. Of these twenty-one isolates were collected from Royal Care International hospital, seventeen isolates were from East Nile hospital and thirty eight isolates were from AL Moalem Medical City. Of the total isolates; thirty six(47.4%) were male and forty(52.6%) were female **(Figure 4.1)**, they were divided into three age groups; seven (9.2%) were less than 25 years, six (7.9%) 25-35 years and sixty three (82.9%) were more than 35 years **(Figure 4.2).**

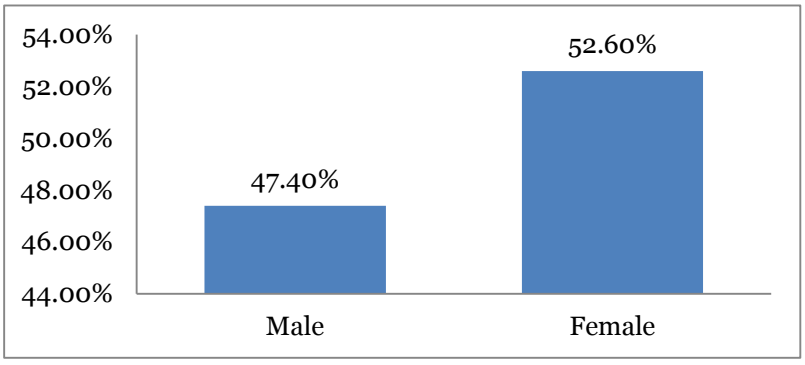


Figure 4.1 Distribution of isolates according to sex

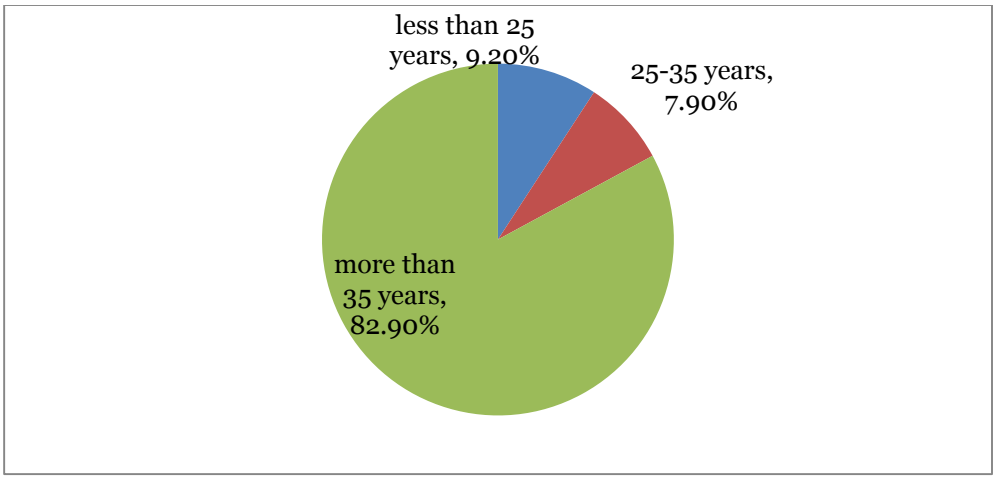


Figure 4.2 Distribution of isolates according to age groups

4.2. Entity of the isolates

The entity of the isolates were as follows; thirty four (44.7%) *K. pnemoniae*, twenty seven (35.5%) *E. coli*, seven (9.2%) *K. oxyltica*, five (6.6%) *P. mirabilis*, one (1.2%) *P. vulgaris* and two (2.6%) 2*Citrobactor* **(Figure 4.3)**

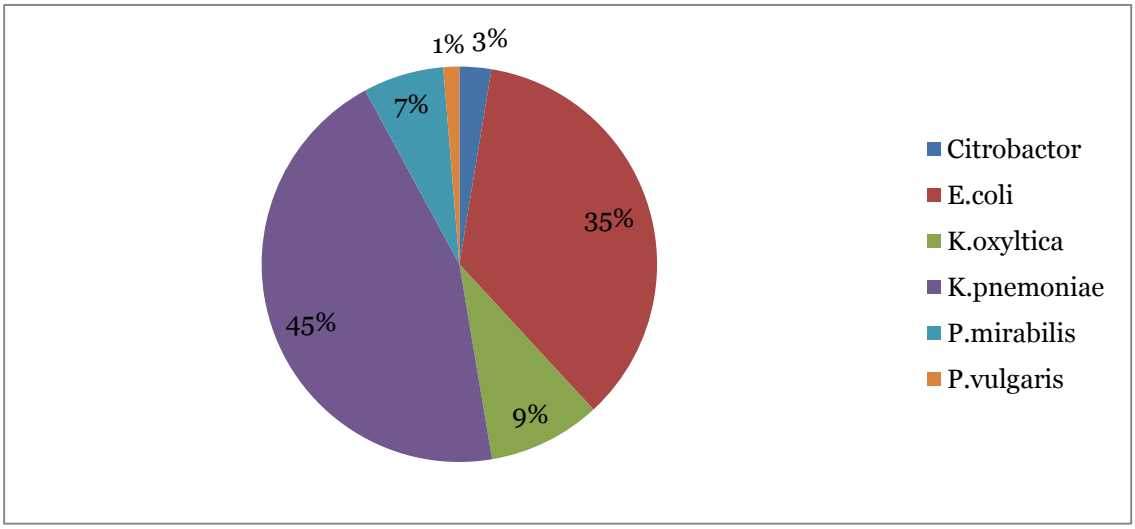


Figure 4.3 Entity of isolates included in the study.

4.3. Types of clinical specimens

Most isolates were from urine specimen forty (52.6%) followed by wound swab twelve (15.8%), sputum seven (9.2%), tissues six(7.9%) and blood five (6.6%) and others as seen in **(Figure 4.4)**.

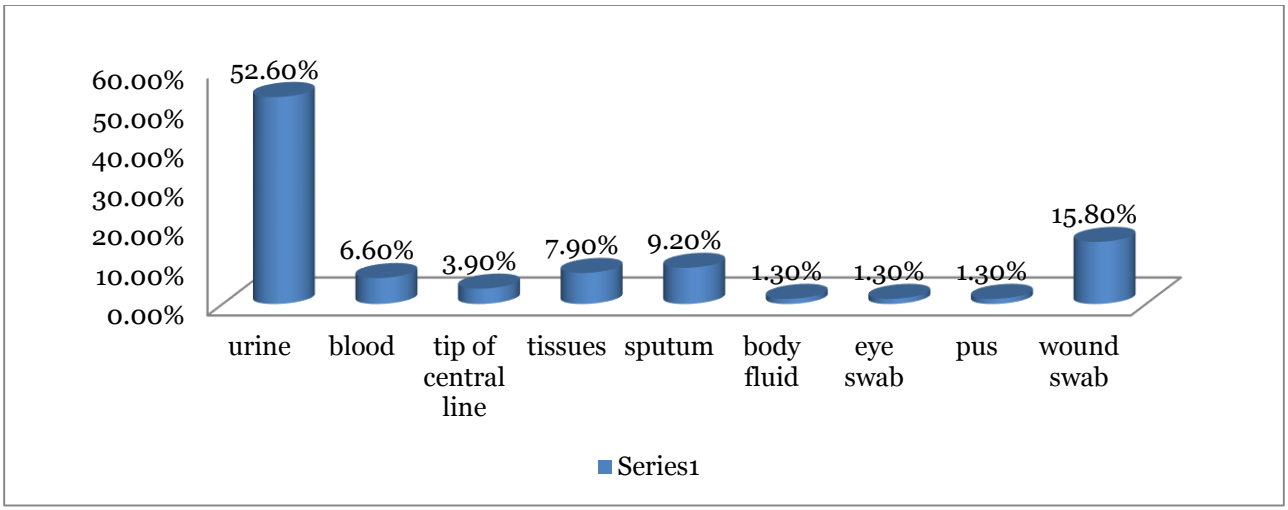


Figure 4.4 Distribution of various isolates according to specimen types

4.4. Results of susceptibility test

Out of seventy-six Enterobacteriaceae isolates twenty nine (38.2%) were seneitive to Cefoxitin and forty seven (61.8%) were resistant, eighteen (23.7%) were seneitive to Ceftazidime and fifty eight (76.30%) were resistant, fifty (65.80%) were seneitive to Chloramphenicol and twenty six (34.2%) were resistant, thirty three (43.4%) were seneitive to Ciprofloxacin and forty three (56.60%) were resistant, fifty (65.8%) were seneitive to Imipenem and twenty six (34.2%) were resistant, fifty four (71.10%) were seneitive to Amikacin and twenty (26.30%) were resistant, sixty two (81.6%) were seneitive to Colistin and fourteen (18.40%) were resistant **(Table 4.1)**.

Three multidrug resistant isolates (resist all antibiotic) were detected, two were *E.coli* isolated from sputam sample of forty two years old male and wound swab of 33 years old male and the third multidrug resistant isolate were *K. oxyltica* isolated from body fluid sample collected from 50 years old female, all this three multidrug resistant isolates were Cefoxitin-cloxacillin double disk synergy (CC-DDS) test positive with no AmpC β-lactamases gene in any of them.

Table 4.1 Results of susceptibility test

Results	Sensitive	Resistant

Antibiotics		
Cefoxitin	38.2%	61.8%
Ceftazidime	23.7%	76.30%
Chloramphenicol	65.80%	34.2%
Ciprofloxacin	43.4%	56.60%
Imipenem	50(65.8%)	34.2%
Amikacin	71.10%	26.30%
Colistin	81.6%	18.40%

4.5. Cefoxitin-cloxacillin double disk synergy (CC-DDS) test

Out of the total isolates nine(22.0%) were found as positives by Cefoxitin-cloxacillin double disk synergy test (CC-DDS); one *K.oxyltica*, one *P.mirabilis*, one *P.vulgaris*, three *E.coli* and three *K.pnemoniae* **(Table 4.2 and figure 4.5)**.

Table 4.2. Distribution of clinical isolates screened positive for Amp C β-lactamases by Cefoxitin-cloxacillin double disk synergy (CC-DDS) test

CXX		Microorganisms					
		Citrobactor	*E.coli*	*K.oxyltica*	*K.pnemoniae*	*P.mirabilis*	*P.vulgaris*
Negative	Count	1	12	4	15	0	0
	% of Total	2.4%	29.3%	9.8%	36.6%	0.0%	0.0%
Positive	Count	0	3	1	3	1	1
	% of Total	0.0%	7.3%	2.4%	7.3%	2.4%	2.4%
Total	Count	1	15	5	18	1	1
	% of Total	2.4%	36.6%	12.2%	43.9%	2.4%	2.4%

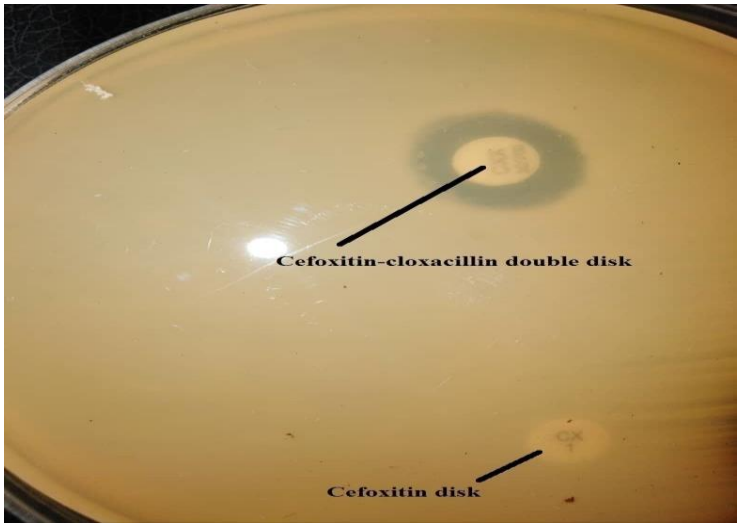


Figure 4.5 Positive Cefoxitin-cloxacillin double disk synergy (CC-DDS) test

4.6. Frequency of AmpC β -lactamases genes groups among the isolates

All seventy six Enterobacteriaceae isolates were investigated for the present of plasmid mediated AmpC β-lactamase genes using multiplex PCR, six(7.9%)of the total isolates were positive for plasmid mediated AmpC β-lactamase genes as follow; three *E.coli*, two *K.pnemoniae* and one *P.vulgaris* **(table 4.3)**

Table 4.3 Frequency of plasmid mediated AmpC β-lactamase genes among the isolates

	GENE	Microorganisms			Total
		E.coli	*K.pnemoniae*	*P.vulgaris*	
CIT	Count	2	2	1	5
	% of Total	33.3%	33.3%	16.7%	83.3%
DHA	Count	1	0	0	1
	% of Total	16.7%	0.0%	0.0%	16.7%
Total	Count	3	2	1	6
	% of Total	50.0%	33.3%	16.7%	100.0%

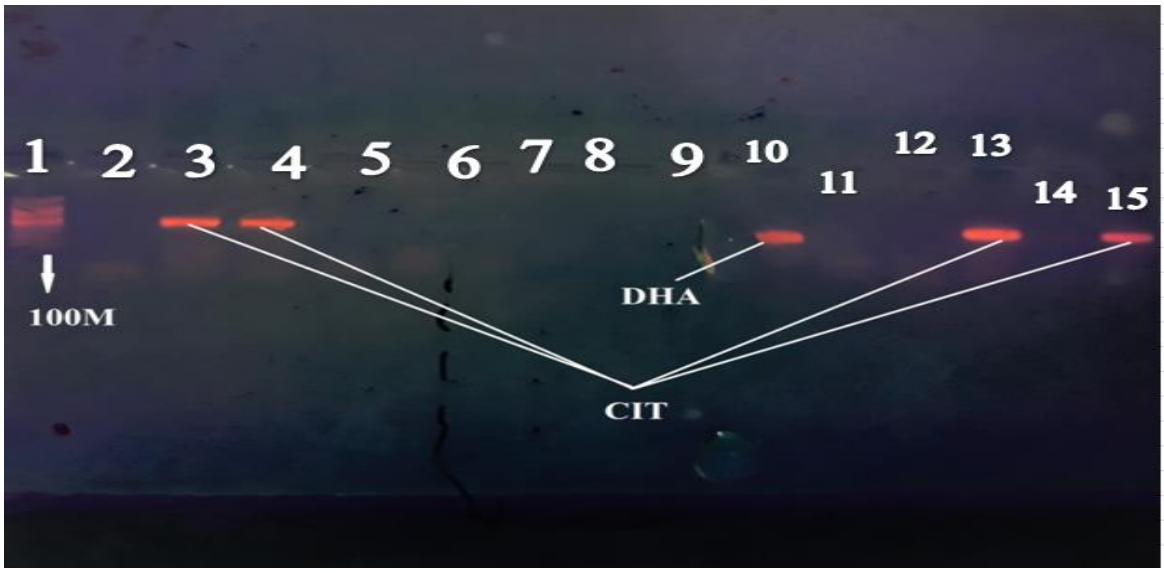


Figure 4.6 Amplified DNA of plasmid mediated AmpC β-lactamase genes. Lane 1, DNA lader 100bp; lane 3, 4, 13 and 15 are typical band size of 462pb corresponding to the molecular size of CIT gene and lane 10 was typical band size of 405pb corresponding to the molecular size of DHAgene.

4.7.Association between plasmid mediated AmpC β-lactamase genes and age groups, gender, clinical specimens and type of organisms

Plasmid mediated AmpC β-lactamase genes showed statistically significant association with the type of the organisms (*p* value= 0.020) and no association between plasmid mediated AmpC β -lactamases genes, age groups, gender and clinical specimens **(Table 4.4).**

Table 4.4 Association between plasmid mediated AmpC β-lactamase genes and age groups, gender, clinical specimens and type of organisms

		AmpC β -lactamases genes		Total	*P* value
Age groups		Present	Absent		
less than 25	Count	1	6	7	0.635
	% of Total	1.3%	7.9%	9.2%	
25 to 35	Count	0	6	6	

	% of Total	0.0%	7.9%	7.9%	
more than 35	Count	5	58	63	
	% of Total	6.6%	76.3%	82.9%	
Gender					
Male	Count	3	33	36	0.893
	% of Total	3.9%	43.4%	47.4%	
Female	Count	3	37	40	
	% of Total	3.9%	48.7%	52.6%	
Clinical specimens					
Urine	Count	1	39	40	0.340
	% of Total	1.3%	51.3%	52.6%	
Blood	Count	1	4	5	
	% of Total	1.3%	5.3%	6.6%	
Tip of central line	Count	0	3	3	
	% of Total	0.0%	3.9%	3.9%	
Tissues	Count	0	6	6	
	% of Total	0.0%	7.9%	7.9%	
Sputum	Count	2	5	7	
	% of Total	2.6%	6.6%	9.2%	
Body Fluid	Count	0	1	1	
	% of Total	0.0%	1.3%	1.3%	
Eye swab	Count	0	1	1	
	% of Total	0.0%	1.3%	1.3%	
Pus	Count	0	1	1	
	% of Total	0.0%	1.3%	1.3%	
wound swab	Count	2	10	12	
	% of Total	2.6%	13.2%	15.8%	
Microorganisms					
Citrobactor	Count	0	2	2	0.020

	% of Total	0.00%	2.60%	2.60%	
E.coli	Count	3	24	27	
	% of Total	3.90%	31.60%	35.50%	
K.oxyltica	Count	0	7	7	
	% of Total	0.00%	9.20%	9.20%	
K.pnemoniae	Count	2	32	34	
	% of Total	2.60%	42.10%	44.70%	
P.mirabilis	Count	0	5	5	
	% of Total	0.00%	6.60%	6.60%	
P.vulgaris	Count	1	0	1	
	% of Total	1.30%	0.00%	1.30%	

CHAPTER V

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

CHAPTER V

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. Discussion

In this study seventy six Enterobacteriaceae isolates were collected from different hospitals in Khartoum state and subculture on MacConky,s ager all isolates were re-identified using biochemical tests, multiplex PCR was used to detect the plasmid mediated AmpC β-lactamase genes; six (7,9%) were detected among nine(22%) of the positive cefoxitin-cloxacillin double disk synergy test (CC-DDS) isolates; one was DHA type gene and five were CIT type gene and there were statistically significant association between the present of AmpC genes and the type of the organisms (*p* value= 0.020), the gene detected in three E. coli, two *K. pneumonia* and one P.vulgaris, the results agreement with; (wassef, *et al*., 2014) reported that 22 AmpC genes were detected in 25.8% of the positive cefoxitin screened isolates of which 40.9% belonged to each of the MOX and the FOX families, 13.6% belonged to the EBC family, and 4.5% belonged to the

CIT family, (Tanushree *et al*., 2013, Cantarelli *et al*., 2007, Wassef *et al*., 2014, Mohamudha *et al*., 2010) that found the predominantly AmpC producers were *Klebsiella pneumonia, E coli,* followed by *Citrobacter spp, Enterobacter spp, Proteus spp* and *Serratia marscence,* also in a study by Soha A El Hady in Egypt; both CMY-1 and CMY-2 were the most common genes detected (Soha A, *et al*., 2015).

Regarding AmpC negative cefoxitin resistance may be attributed to ESBLs and MBL production or non- enzymatic mechanism such as porin channel mutation (Subbalakshmi *et al*., 2016), over expression of chromosomal AmpC gene due to mutation in the promoter or attenuator regions (Subbalakshmi *et al*., 2016), the other reason is that cefoxitin has been demonstrated as a substrate to active efflux pump in clinical isolates (Subbalakshmi *et al*., 2016).

5.2. Conclusion

In this study the most prevalent AmpC genes belonged to CIT and DHA, AmpC β-lactamases producing Enterobacteriaceae are being frequently isolated from clinical samples, as they increase the complexity of their detection, in addition, this study demonstrates the useful of multiplex PCR method is reliably identification of plasmid mediated AmpC β-lactamase genes.

5.3. Recommendations

Identifying the types of AmpC may aid in hospital infection control and help the physician to prescribe the most appropriate antibiotic, thus decreasing the selective pressure, which generates antibiotic resistance. Sequencing and typing the strains may be required to better understand the genetic relatedness and the molecular epidemiology of their resistance mechanism. Continual surveillance of resistance mechanisms that assist appropriate antibiotic therapy and better patient outcomes and also reduce antibiotic resistance through better infection control practices.

REFERENCEA

1. Abraham, E. P. and Chain, E. (1940). An enzyme from bacteria able to destroy penicillin. *Nature,* 146(3713), 837-837.
2. Alksne, L. E. and B. A. Rasmussen. (1997). Expression of the AsbA1, OXA-12, and AsbM1 β-lactamases in *Aeromonas jandaei* AER 14 is coordinated by a two-component regulon. J. *Bacteriol*, 179:2006–2013
3. Ambler, R. P. (1980). The structure of β-lactamases. Philosophical Transactions of the Royal Society of London. *B, Biological Sciences*, 289(1036), 321-331.
4. Anitha Madhavan, V Jayalakshmi.(2016) Occurrence of extended-spectrum betalactamase, AmpC and MBLase producers among multidrug-resistant Enterobacteriaceae causing urinary tract infection in a tertiary health-care teaching hospital. *Journal of The Academy of Clinical Microbiologists*, 18(2): 80-85.

5. Avison, M. B., P. Niumsup, K. Nurmahomed, T. R. Walsh, and P. M. Bennett. (2004).Roleofthe‘cre/blr-tag’DNA sequencein regulation of gene expression by the *Aeromonas hydrophila* β -lactamase regulator, BlrA. *J. Antimicrob. Chemother*. 53:197–202
6. Bauernfeind, A., Chong, Y., & Lee, K. (1998). Plasmid-encoded AmpC β-actamases: how far have we gone 10 years after the discovery?. *Yonsei medical journal*, 39(6), 520-525.
7. Bell, M., Chitsaz, D., Turnidge, M., Barton, L., Walters and Jones. (2007). Prevalence and significance of a negative extended-spectrum β-lactamase (ESBL)
8. Bergstro¨m, S., O. Olsson, and S. Normark. (1982). Common evolutionary origin of chromosomal beta-lactamase genes in Enterobacteria. *J.Bacteriol.* 150:528–534.).
9. Bolmstro¨m, A., A. Engelhardt, L. Bylund, P. Ho, and Å. Karlsson. (2006). Evaluation of two new Etest strips for AmpC detection, abstr. D-0451. Abstr. 46th Intersci. Conf. *Antimicrob. Agents Chemother*
10. Bou, G., and J. Martinez-Beltran. (2000). Cloning, nucleotide sequencing, and analysis of the gene encoding an AmpC β-lactamase in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother*. 44:428–432
11. Bush. (1989). Characterization of beta-lactamases. *Antimicrobial agents and chemotherapy*, 33(3), 259.
12. Bush, K., Jacoby, G. A., and Medeiros, A. A. (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. *Antimicrobial agents and chemotherapy*, 39(6), 1211.
13. Cantarelli, V. V., Inamine, E., Brodt, T. C. Z., Secchi, C., Cavalcante, B. C., and Pereira, F. D. S. (2007). Utility of the ceftazidime-imipenem antagonism test (CIAT) to detect and confirm the presence of inducible AmpC β-lactamases among Enterobacteriaceae. *Brazilian Journal of Infectious Diseases*, 11(2), 237-239.
14. Chast, F. (2008). A history of drug discovery: from first steps of chemistry to achievements in molecular pharmacology. *In The Practice of Medicinal Chemistry* (pp. 1-62). Academic Press.
15. Chow, JW., Fine, MJ., Shlaes, DM., Quinn, JP., Hooper, DC., Johnson, MP., Ramphal, R., Wagener, MM., Miyashiro, DK and Yu, VL. (1991) *Enterobacter bacteremia*: clinical features and emergence of antibiotic resistance during therapy. *Ann. Intern. Med.*; 115: 585-590.

16. Cole, K. (1986). Inducible cephalosporinase production in clinical isolates of *Enterobacter cloacae* is controlled by a regulatory gene that has been deleted from *Escherichia coli*. *EMBO J*. 5:3709–3714
17. Coudron, PE., Moland, ES., and Thomson KS. Occurrence and detection of Amp C β lactamase among *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* isolates at a veterans Medical Center. J Clin Microbiol. 2000; 1791-6.
18. Denyer, S. P., and Maillard, J. Y. (2002). Cellular impermeability and uptake of biocides and antibiotics in Gram- negative bacteria. *Journal of applied microbiology*, 92, 35S-45S.
19. Clinical and Laboratory Standards Institute(CLSI-15), Performance Standards for Antimicrobial Susceptibility Testing. Approved Standards M100-S25
20. Doi, Y., and D. L. Paterson. (2007). Detection of plasmid-mediated class C β-lactamases. *Int. J. Infect. Dis*. 11:191–197
21. Elliott, W., and Chan, J. (2017). Meropenem and Vaborbactam Injection (Vabomere). *Internal Medicine Alert*, 39(20)).
22. Faiyaz, M., Gupta, S., and Gupta, D. (2019). Antibiotic against gram-negative bacteria. High Value Fermentation Products: *Human Health*, 79-101.
23. Gupta, N., Limbago, B. M., Patel, J. B., and Kallen, A. J. (2011). Carbapenem-resistant Enterobacteriaceae: epidemiology and prevention. *Clinical infectious diseases*, 53(1), 60-67
24. Hanson, ND., Moland, ES., Hong, SG., Propst, K., Novak, DJ and Cavalieri SJ. (2008) Surveillance of community-based reservoirs reveals the presence of CTX M, imported AmpC, and OXA-30 β-lactamases in urine isolates of Klebsiella pneumoniae and Escherichia coli in a U.S. community. *Antimicrob. Agents Chemother*, 52:3814-3816.
25. Higgins, CS., Avison, MB., Jamieson, L., Simm, AM., Bennett, PM and Walsh, TR. (2001). Characterization, cloning and sequence analysis of the inducible Ochrobactrum anthropi AmpC β-lactamase. *J. Antimicrob. Chemother*, 47:745-754.
26. Inghal, S., Mathur, T., Khan, S., Upadhyay, D. J., Chugh, S., Gaind, R., and Rattan, A. (2005). Evaluation of methods for AmpC β-lactamase in Gram negative clinical isolates from tertiary care hospitals. *Indian journal of medical microbiology*, 23(2), 120)
27. Inverarity, D. (2010). Oxford handbook of infectious diseases and microbiology. *Journal of Hospital Infection*, 75(2), 148.
28. Jacoby, G. A. (2009). AmpC β-lactamases. *Clinical microbiology reviews*, 22(1), 161-182.

29. Jaurin, B., Grundstro¨m, Edlund, and Normark. (1981). The *E. coli* β-lactamase attenuator mediates growth rate-dependent regulation. *Nature*, 290:221–225.
30. Jorgensen, J. H., Hindler, J. F., Reller, L. B., and Weinstein, M. P. (2007). New consensus guidline from the Clinical and Laboratory Standard Institute for antimicrobial susceptibility testing of infrequently isolated or fastidious bacteria. *Clinical infectious disease,* 44(2), 280-286.
31. Juan, C., M. D. Macia, O. Gutierrez, C. Vidal, J. L. Perez, and A. Oliver. (2005). Molecular mechanisms of β-lactam resistance mediated by AmpC hyperproduction in *Pseudomonas aeruginosa* clinical strains. *Antimicrob. Agents Chemother*. 49:4733–4738
32. Juan, C., B. Moya´, J. L. Pe´rez, and A. Oliver. (2006). Stepwise upregulation of the *Pseudomonas aeruginosa* chromosomal cephalosporinase conferring high-level β-lactam resistance involves three AmpD homologues. *Antimicrob. Agents Chemother*. 50:1780–1787
33. Knott-Hunziker, V., Petursson, S., Gayatilake, GS., Waley, SG., Jaurin, B and Grundström T. (1982). Active sites of β-lactamases. *Biochem. J,* 201:621627.
34. Knox JR, Moews PC, Frere JM. (1996). Molecular evolution of bacterial β-lactam resistance. *Chem. Biol*, 3:937-47.
35. Kong, K. F., S. R. Jayawardena, S. D. Indulkar, A. Del Puerto, C. L. Koh, N. Hoiby, and K. Mathee. (2005). *Pseudomonas aeruginosa* AmpR is a global transcriptional factor that regulates expression of AmpC and PoxB β-lactamases, proteases, quorum sensing, and other virulence factors. *Antimicrob. Agents Chemother*. 49:4567–4575.).
36. Livermore, D. M. (1995). β-Lactamases in laboratory and clinical resistance. *Clinical microbiology reviews*, 8(4), 557-584.
37. Livermore, M., and Brown, F., (2001). Detection of β-lactamase-mediated resistance. *Journal of antimicrobial Chemotherapy*, 48(suppl_1), 59-64.)
38. Li Y, Li Q, Du Y, Jiang X, Tang J, Wang J, et al. (2008). Prevalence of plasmid mediated AmpC β-lactamases in a Chinese university hospital from 2003 to 2005: first report of CMY-2-type AmpC β-lactamase resistance in China. *J Clin Microbiol*, 46:1317–21.
39. Maha Dirar, Naser Bilal, Mutasim E. Ibrahim and Mohamed Hamid. (2020). Resistance Patterns and Phenotypic detection of β-lactamase enzymes among Enterobacteriaceae isolates from referral hospitals in Khartoum state, Sudan. *Cureus* 12(3).

40. Mahlen, S. D., S. S. Morrow, B. Abdalhamid, and N. D. Hanson. (2003). Analyses of ampC gene expression in *Serratia marcescens* reveal new regulatory properties. *J. Antimicrob. Chemother*. 51:791–802
41. Migliavacca, R., E. Nucleo, M. M. D'Andrea, M. Spalla, T. Giani, and L. Pagani. (2007). Acquired AmpC type β-lactamases: an emerging problem in Italian long-term care and rehabilitation facilities. *New Microbiol*. 30: 295–298
42. Mohamudha P, Harish B and Parija S. (2010) AmpC β-lactamases among Gram negative clinical isolates from a tertiary hospital, South India. *Brazilian Journal of Microbiology*. 41(3):596-602
43. Moland E, Hanson N, Black J, Hossain A, Song W and Thomson K. (2006), Prevalence of newer β-lactamases in Gram-negative clinical isolates collected in the United States from 2001 to 2002. *J Clin Microbiol*, 44(9):3318–32.
44. Moya, B., C. Juan, J. Blazquez, L. Zamorano, J. L. Perez, A. Oliver, and H. Son Dureta. (2008). Abstr. 48th Intersci. Conf. Antimicrob. *Agents Chemother., abstr*, C1-3729Miriagou, V., P. T. Tassios, N. J. Legakis, and L. S. Tzouvelekis. (2004). Expanded-spectrum cephalosporin resistance in non-typhoid *Salmonella. Int. J. Antimicrob. Agents* 23:547–555
45. Munoz-Price, S., Poirel, L., Bonomo, R., Schwaber, J., Daikos, L., Cormican, M., and Quinn, J. P. (2013). Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. *The Lancet infectious diseases*, 13(9), 785-796.
46. Mutasim, E., Mohammed, A., Abdullah, M., and Bahaeldin, K. (2019). Phenotypic characterization and antibiotic resistance patterns of extended-spectrum β-Lactamase- and AmpC β-lactamase-producing Gram-Negative bacteria in a Referral hospital, Saudi Arabia. *Canadian Journal of Infectious Diseases and Medical Microbiology*
47. Nathwani, D., and Wood, M. J. (1993). Penicillins. Drugs, 45(6), 866-894.
48. Neu, H. C. (1985). Relation of structural properties of β-lactam antibiotics to antibacterial activity. *The American journal of medicine*, 79(2), 2-13
49. Nikaido, H., W. Liu, and E. Y. Rosenberg. (1990). Outer membrane permeability and β-lactamase stability of dipolar ionic cephalosporins containing methoxyimino substituents. *Antimicrob. Agents Chemother*. 34:337–342
50. Normark, B. H., and Normark, S. (2002). Evolution and spread of antibiotic resistance. *Journal of internal medicine*, 252(2), 91-106.

51. Ogata H, Renesto P, Audic S, Robert C, Blanc G, Fournier PE, Parinello H, Claverie JM and Raoult, D. (2005). The genome sequence of Rickettsia felis identifies the first putative conjugative plasmid in an obligate intracellular parasite. *PLoS Biol*; 3:248
52. Pai, K., Pischon, T., Ma, J., Manson, J. E., Hankinson, S. E., Joshipura, K., and Rimm, E. B. (2004). Inflammatory markers and the risk of coronary heart disease in men and women. *New England Journal of Medicine*, 351(25), 2599-2610
53. Papanicolaou, G. A., Medeiros, A., and Jacoby, G. (1990). Noval plasmid- mediated beta-lactamase(MIR-1) conferring resistance to oxyimino-and alpha-methoxy beta-lactams in clinical isolates of *Klebsiella pneumonia. Antimicrobial agents and chemotherapy*, 34(11), 2200-2209.
54. Paterson, D. (2006). Resistance in Gram-negative bacteria: Enterobacteriaceae. *Americ an journal of infection control*, 34(5), S20-S28.
55. Pérez-Pérez, J., and Hanson, D. (2002). Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *Journal of clinical microbiology*, 40(6), 2153-2162. .
56. Philippon, A., Arlet, G., and Jacoby, A. (2002). Plasmid-determined AmpC-type β-lactamases. *Antimicrobial agents and chemotherapy*, 46(1), 1-11.
57. Pitout, J. (2012). Extraintestinal pathogenic *Escherichia coli*: a combination of virulence with antibiotic resistance. *Frontiers in microbiology*, 3, 9.
58. Quale, J., S. Bratu, J. Gupta, and D. Landman. (2006). Interplay of efflux system, ampC, and oprD expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. *Antimicrob. Agents Chemother*. 50:1633–1641.
59. Rice, B., Willey, H., Papanicolaou, A., Medeiros, A., Eliopoulos, M., Moellering, C., and Jacoby, A. (1990). Outbreak of ceftazidime resistance caused by extended-spectrum β-lactamases .
60. Rodríguez-Baño, J., Navarro, M. D., Retamar, P., Picón, E and Pascual, Á, (2012). Extended-Spectrum BetaLactamases–Red Española de Investigación en Patología Infecciosa/Grupo de Estudio de Infección Hospitalaria Group. β-Lactam/β-lactam inhibitor combinations for the treatment of bacteremia due to extended-spectrum β-lactamase–producing *Escherichia coli*: a post hoc analysis of prospective cohorts. *Clinical Infectious Diseases*, 54(2), 167-174.

61. Ruppé, É., Woerther, P. L., & Barbier, F. (2015). Mechanisms of antimicrobial resistance in Gram negative bacilli. *Annals of intensive care*, 5(1), 1-15.
62. Sarah M. Drawz, and Robert A. (2010). Clin. Microbiol. Rev.; doi:10.1128/CMR.00037-09
63. Shahid, M., Malik, A., Agrawal, M., and Singhal, S. (2004). Phenotypic detection of extended-spectrum and AmpC β-lactamases by a new spot-inoculation method and modified three-dimensional extract test: comparison with the conventional three-dimensional extract test. *Journal of Antimicrobial Chemotherapy*, 54(3), 684-687.)
64. Singh, B., and Barrett, F. (2006). Empirical antibacterial drug discovery foundation in natural products. *Biochemical pharmacology*, 71(7), 1006-1015.
65. Soha, A., El-Hady, Lamiaa, A., and Adel. (2015). Occurrence and detection of AmpC blactamases among Enterobacteriaceae isolates from patients at Ain Shams University Hospital. *The Egyptian Journal of Medical Human Genetics* , 16:239–244.
66. Song W, Kim J and Kim H. (2006). Increasing trend in the prevalence of plasmid-mediated AmpC β-lactamases in Enterobacteriaceae lacking chromosomal ampC gene at a Korean university hospital from 2002 to 2004. *Diagn Microbiol Infect* Dis;55:219–24.
67. Spratt, G. (1994). Resistance to antibiotics mediated by target alterations. *Science*, 264(5157), 388-393.
68. Stock, I., Sherwood, KJ and Wiedemann B. (2003). Natural antibiotic susceptibility of Ewingella americana strains. *J. Chemother*, 15:428-441.
69. Suarez, C., and Gudiol, F. (2009). β-lactam antibiotics. *Enfermedades infecciosas y microbiologia clinical*, 27(2), 116-129.
70. Su, L., Chia, C., Chu, A., Kuo and Chiu. (2005). Increasing ceftriaxone resistance in *Salmonella* isolates from a university hospital in Taiwan. *J. Antimicrob. Chemother*. 55:846–852.
71. Subbalakshmi, E., Ranjani and Cand, R. (2016) A study on detection of extended-spectrum β-lactamases (ESBLs) and comparison of various phenotypic methods of AmpC detection in *Pseudomonas aeruginosa* from various clinical isolates in a tertiary care teaching hospital. *Mueller J Med Science & Research*, 7(1):35-9
72. Sykes, B., Bonner, P., Bush, K., Georgopapadakou, H., and Wells, S. (1981). Monobactams — monocyclic β-lactam antibiotics produced by bacteria. *Journal of Antimicrobial Chemotherapy*, 8(suppl_E), 1-10.

73. Tan, T., Ng, S., Teo, L., Koh, Y and Teok C. (2008). Detection of plasmid mediated AmpC in *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis*. *J Clin Path*, 61:642–4.
74. Tanushree, Barua., Malini, Shariff and S.S.Thukral. (2013). Detection and characterization of AmpC -lactamases in Indian clinical isolates of *Escherichia coli, Klebsiella pneumoniae* and *Klebsiella oxytoca*. *Universal J. Microbiol*. Res.,2013: 1(2):15
75. Then, R. L., and P. Angehrn. (1982). Trapping of nonhydrolyzable cephalosporins by cephalosporinases in *Enterobacter cloacae* and *Pseudomonas aeruginosa* as a possible resistance mechanism. *Antimicrob. Agents Chemother*. 21:711–717
76. Thomson, K. S. (2001). Controversies about extended-spectrum and AmpC β-lactamases. *Emerging infectious diseases*, 7(2), 333.
77. Varun, G., Sumati, A and Kardesai. (2013). Prevalence of extended-spectrum beta-lactamases, AmpC beta- lactamase, and metallo- beta- lactamase producing *Pseudomonas aeruginosa* and *Acinetobacter*. *Journal of the Scientific Society*, 40(1). 28-31
78. Wassef M, Behiry I, Younan M, El Guindy N, Mostafa S and Abada E. (2014). Genotypic Identification of AmpC β-Lactamases Production in Gram-Negative Bacilli Isolates. *Jundishapur Journal of Microbiology*, 7
79. Winn WC, Koneman EW (2006). Koneman's color atlas and textbook of ed. diagnostic microbiology. *6th Philadelphia: Lippincott Williams & Wilkins*.
80. Yan JJ, Ko WC, Wu JJ, Tsai SH, Chuang CL. (2004). Epidemiological investigation of bloodstream infections by extended spectrum cephalosporin-resistant *Escherichia coli* in a Taiwanese teaching hospital. *J. Clin. Microbiol*, 42:3329-3332
81. Yan J, Hsueh P, Lu J, Chang F, Shyr J and Wan J. (2006). Extended spectrum β-lactamases and plasmid-mediated AmpC enzymes among clinical isolates of *Escherichia coli* and *Klebsiella pneumonia* from seven medical centers in Taiwan. *Antimicrob Agents Chemother*, 50:1861–4
82. Yagi, T., Wachino, J., Kurokawa, H., Suzuki, S., Yamane, K., Doi, Y., and Arakawa, Y. (2005). Practical methods using boronic acid compounds for identification of class C β-lactamase producing *Klebsiella pneumoniae* and *Escherichia coli*. *Journal of Clinical Microbiology*, 43(6), 25512558

83. Yamasaki, K., Komatsu, M., Abe, N., Fukuda, S., Miyamoto, Y and Higuchi, T. (2010). Laboratory surveillance for prospective plasmid mediated AmpC β-lactamases in the Kinki region of Japan. *J Clin Microbiol*, 48:3267–73.

Appendix I – questionnaire

Sudan University of Science and Technology

College of Graduate studies

Detection of Plasmid Mediated AmpC β-Lactamase Genes in Enterobacteriaceae Isolates from Hospitalized Patients in Khartoum State

By : Anfal Ibrahim Bahereldeen Adam

Supervisor: Prof. Humodi A. Saeed

Hospital name: ……

Sample NO: ……Type of specimen ……

Specimen data: ……

Patient code: ……gender: ……age: ..……

Lablotery result

1. Isolated microorganisms

   ……

2. Sensitivity results

Sensitive to:

……..……..……

resist to:

……

3. Cefoxitin-cloxacillin double disk synergy test (CC-DDS)results
4. ……..
5. Multiplex PCR result
6. ……

Appendix II

Reagents and Stains

Gram Stain (Cheesebrough, 2000)

Requirements

Crystal violet Gram stain (Hi Media)

To make 1 liter

Crystal violet…… ... ... …20 g

Ammonium oxalate…… .....……9 g

Ethanol or methanol, absolute…… .....… ....….95 g

Distilled water…… to 1 liter

lugol's iodine (Hi Media)

To make 1 liter

Potassium iodide…… .....……20 g

Iodine……..10 g

Distilled water…….. to 10 liter

70% alcohol

Absolute alcohol…… .....…..70 ml

Distilled water…… .....…….30 ml

Safranin (HiMedia)

Turbidity Standard

1% v/v solution of sulpharic acid was prepared by adding 1 ml of concentrated sulfuric acid to 99 ml of water. Mix well.

1.17% w/v solution of barium chloride was prepared by dissolving of 2.35g of dehydrated barium chloride (BaCl2.2H2O) in 200 ml of distilled water.

To make the turbidity standard 0.5 ml of barium chloride solution was added to 99.4 ml of the sulpharic acid solution and mix well.

A small volume of the turbid solution was transferred to screw caped bottle of the same types as used for preparing the test and control inoculate (Chemie, 2014).

Physiological saline (0.85% w/v)

Sodium chloride	8.5 g
Distilled water	1 L

Preparation

By dissolving 8.5 g of NaCl in 1000 ml distilled water in beaker. Then the beaker was covered with foil and Sterilized by autoclaving at 15 Ibs pressure (121°C) for15 minutes.

Appendix III

Preparation of Media (Chemie , 2014)

All media are prepared from Himedia Laboratories pvt, Ltd, made in India.

MacConkey agar

Formula in grams per liter

Peptone	3.00
Pancreatic digest of gelatin	17.00
Lactose monohydrate	10.00
Bile salts	1.500
Sodium chloride	5.00
Neutral red	0.03
Agar	13.50

Preparation

By dissolving 49.53 grams of medium in 1000 ml distilled water. The medium was sterilized by autoclaving at 15 Ibs pressure (121°C) for15 minutes, cooled and poured in petridishes.

Kligler Iron Agar

Formula in grams per liter

Peptone mixture 20,00

Sodium Chloride..... 5,00

Ferric Ammonium Citrate 0,50

Phenol Red 0,025

Lactose 10,00

Dextrose 1,00

Bacteriological Agar 15,00

Sodium Thiosulfate.....0,50

Preparation

Suspend 52 grams of the medium in one liter of distilled water. Mix well and heat with frequent agitation. Boil for one minute. Dispense into tubes and sterilize at 121º C (15lbs. pressure) for 15 minutes. Allow to cool in a slanted position so as to obtain butts of 1'5-2 cm. Depth. For greater accuracy, Kligler Iron Agar should be used on the day of preparation or melted and solidified before use.

Tryptophan Culture Broth

Formula in grams per liter (PH 7.5)

Casein Peptone 10,00

L-Tryptophan 1,00

Sodium chloride.....5,00

Preparation

Suspend 16,0 grams of medium in one liter of distilled water. Heat to boiling agitating frequently. Distribute in test tubes, 3 ml each. Close the tubes with cotton or with a plastic or metallic cap. Sterilize at 121º C (15 lbs. sp.) for 15 minutes.

Simmons Citrate Agar

Formula in grams per liter (PH 7)

Ammonium Dihydrogen Phosphate 1,00

Dipotassium Phosphate..... 1,00

Sodium Chloride.....5,00

Sodium Citrate 2,00

Magnesium Sulfate.....0,20

Bacteriological Agar..... 15,00

Bromthymol Blue.....0,08

Preparation

Suspend 24,3 grams of the medium in one liter of distilled water. Mix well and heat with frequent agitation until completely dissolved. Dispense in tubes and sterilize in the autoclave at 121ºC (15 lbs sp.) for 15 minutes. Cool the tubes in a slanted position so that the base is short (1-1,5 cm. deep). Alternatively, the media can be poured into petri plates.

Christensen's Urea Agar

Formula in grams per liter (PH 6.9)

Gelatin Peptone..... 1,00

Dextrose 1,00

Sodium Chloride.....5,00

Monopotassium Phosphate 2,00

Urea 20,00

Phenol Red.....0,012

Preparation

Dissolve 29 grams of the medium in 100 ml. of distilled water. Sterilize by filtration. Separately dissolve 15 grams of agar in 900 ml. of distilled water by boiling. Sterilize in autoclave at 121°C (15 lbs.sp) for 15 minutes. Cool to 50°C and add to the 100 ml. of the sterile Urea Agar Base.

Mix well and dispense aseptically in sterile tubes. Leave the medium to set in a slanted position so as to obtain deep butts. At a pH of 6.8 to 7.0 the solidified medium should have a light pinkish yellow colour. Do not remelt the slanted agar.

Mueller-Hinton Agar

Formula in grams per liter (PH 7.4)

Beef, infusion …….300.0g

Cas amino acids……17.5 g

Starch……..1.5g

Agar …….17.0g

Distilled water…….1000ml

Preparation

38.0 g of media was suspended in 100 ml distilled water. Sterilized by autoclaving at 15Ib pressure (121˚C) and poured in sterile petri dishes.

Nutrient agar

Formula in grams per liter (PH 7.4)

Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00

Final pH 7.4 ± 0.2 at 25 °C

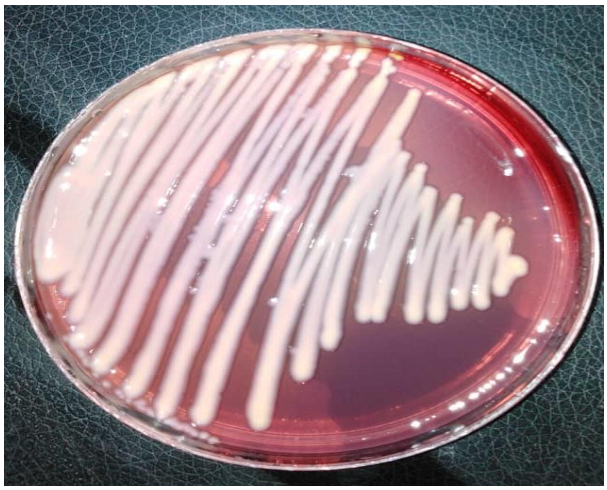
Preparation

By dissolving 28 g of powder in 1000 ml distilled water. The medium was sterilized by autoclaving at 15 Ibs pressure (121°C) for15 minutes, cooled and poured in petridishes

Appendix VI

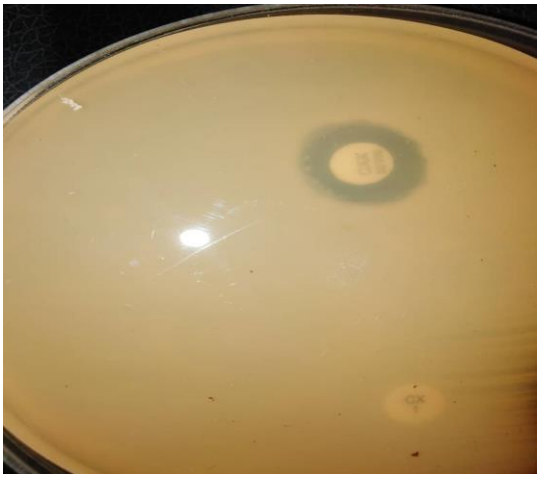
Color plates

Color plate 1.1 Incubator device



Color plate 1.2. *Klebsiella spp* on MacConkey agar media showing pink color indicating lactose fermentation.

Color plate 1.3. Biochemical test of *P.mirabilis*



Color plate 1.4. Positive Cefoxitin-cloxacillin double disk synergy (CC-DDS) test

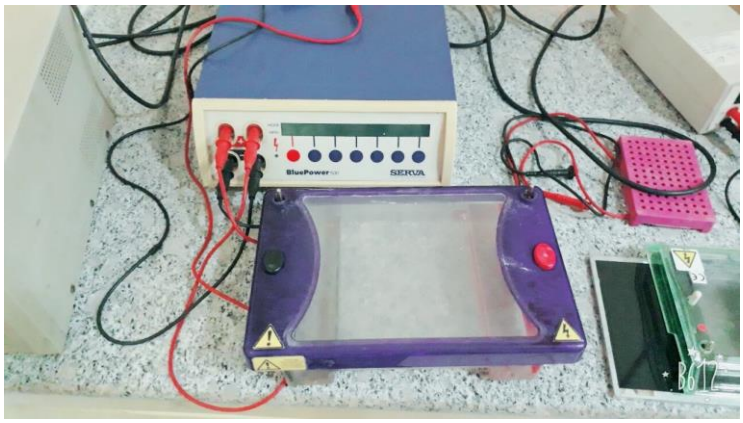
Color plate 1.5. DNA extraction reagents (guanidine chloride method)

Color plate 1.6. Microcenterfuge device

Color plate 1.7. AmpC β-lactamases . Primers specific for the genes of six different phylogenetic Groups (bla MOX, bla CIT, bla DHA, bla ACC, bla EBC, and bla FOX) were ordered from Macrogen Company, Korea

Color plate 1.8. Thermocycle device

Color plate 1.9. PCR protocol



Color plate 1.10. gel electrophoresis and power supply device