Addis Ababa University
College of Health Sciences
Department of Medical Laboratory Sciences

Comparison of four phenotypic assays for the detection of ESBL producing gram negative bacilli and Assessment of the magnitude of carbapenemase-producing gram-negative bacilli isolated from specimens referred to Ethiopian Public health Institute.

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This is to certify that the thesis prepared by **Abera Abdeta**, entitled: **Comparison of four phenotypic assays for the detection of ESBL producing gram negative bacilli and Assessment of the magnitude of carbapenemase-producing gram-negative bacilli isolated from specimens refrred to Ethiopian Public health Institute**and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology Specialty) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abbreviations

AMR:----------Antimicrobial resistance

ASLM: ----------African Society for Laboratory Medicine

AST:---------- Antimicrobial susceptibility testing

ATCC:----------American Type Culture Collection

Bla:---------- β-lactamases

CAL:----------Ceftazidime+Clavulanic acid

CAZ:---------- Ceftazidime

CDC:----------Centers for Disease Control and Prevention

CLSI:----------Clinical and LaboratoryStandardsInstitute

CRE:----------carbapenem-resistant Enterobacteriaceae

CTL:----------Cefotaxime+Clavulanic acid

CTX:----------Cefotaxime

CTX-M:----------Cefotaximases

EARS-NeT----------European Antimicrobial Resistance Surveillance Network

ECDC---------- European Centre for Disease Prevention and Control

EDTA----------Ethylenediaminetetraacetic acid

ENAO----------Ethiopian National Accreditation Office

EPHI----------Ethiopian Public Health Institute

ESBL----------Extended Spectrum Beta-lactamase

ESBL-E----------Extended Spectrum Beta-lactamase producing Enterobacteriaceae

EUCAST----------European Committee on Antimicrobial Susceptibility Testing

GDP----------Gross domestic product

GNB----------Gram-negative bacilli

HCWs----------Health care workers

ICARE----------Intensive Care Antimicrobial Resistance Epidemiology

IMP----------ImipenemaseMetallo-β-lactamases

ISO----------International Organization for Standardization

JUSH----------Jimma University Specialized Hospital

KPC----------*KlebsiellaPneumoniae*Carbapenemase

MBL----------Metallo β-lactamase

MBO----------Meropenem+Phenylboronic acid

mCIM----------Modified Carbapenem Inactivation Method

MDR----------Multidrug resistance

MDROs----------multidrug-resistant organisms

MHA----------Muller Hinton Agar

MIC----------Minimum Inhibitory Concentration

MRP----------Meropenem

NDM-1----------New Delhi Metallo-β-lactamase-1

NFGNB---------- Non-fermentative gram-negative bacilli

OXA----------Oxacilinase

sCIM----------simplified Carbapenem Inactivation Method

SHV----------sulphydryl variable

SLIPTA----------Stepwise Laboratory Quality Improvement Process towards Accreditation

SOP----------Standard operating procedures

SPSS----------Statistical package for social sciences

TEM----------Temoniera

US $----------United States of America dollars

USA----------United States of America

UTI----------Urinary Tract Infections

VIM:----------Verona integron-encoded Metallo-β-lactamase

µg----------microgram

Abstract

Background*:* The detection of extended-spectrum β-lactamase producing (ESBL) *Enterobacteriaceae* and carbapenemase-producing gram-negative bacilli are of importance for infection control and epidemiological purposes. The common mechanism of resistance to extended spectrum cephalosporins and carbapenem antibiotics is through the production of ESBL and carbapenemase enzymes. Thus, this study was designed to compare four phenotypic assays to detect ESBLs in clinical isolates of *Enterobacteriaceae* and determine the magnitude of carbapenemase-producing gram-negative bacilli.

Objective:To compare four phenotypic assays for the detection of ESBL production and to determine the magnitude of carbapenemase-producing gram-negative bacilli.

Methods:The study was conducted from June 30, 2019, to May 30, 2020. Modified and simplified carbapenem inactivation methods were used to screen for carbapenemase. The MICand combination disk methods were used to confirm *Klebsiella pneumonia* carbapenemase (KPC) and Metallo β-lactamases (MBL) respectively. ESBL detectionassays (Vitek2 Compact, ESBL Etests, combination disks, DDS methods on Mueller-Hinton [MHA]) were compared. Initially, data were entered on WHOnet 2019 and analyzed using both WHOnet 2019 and SPSS 23 version.

Results:Out of 117 clinical isolates, CAZ/CAL disk, CTX/CTL disk, DDS, Vitek 2 compact, MIC CAZ/CAL, and MIC CTX/CTL detected, 65% (76/117), 64.1% (75/117),64.1% (75/117), 63.2% (74/117), 60.7% (71/117), and 59.8% (70/117) ESBL producers respectively.Out of 429 clinical isolates, 15.4% (66/429), 5.4% (23/429), and 23.77% (102/429 isolates were non-susceptible to carbapenems, carbapenem ase positive, and MDR respectively.0.7% (3/429) and 2.3% (10/429) were positivefor KPC and MBL respectively. The highest and lowest resistance was observed against Ampicillin (92%) and Amikacin (11.2%) respectively.

Conclusions: The prevalence of MDR, carbapenem non susceptible and carbapenemase producing gram negative bacilli was found to be high in this study. In this study, a combination disk method was found to be superior to other methods for ESBL detection. Metallo β-lactamase was found to be the most prevalent carbapenemase among clinical isolates. For prevention infection control and regular surveillance must be enhanced.

Keywords: Extended-spectrum β-lactamase, Carbapenemase,Modified carbapenem inactivation method,Combination disk method, Ethiopia

1. Introduction

1.1. Background

All β-lactam antimicrobial agents share a central, four-member β-lactam ring and bind to at least two penicillin-binding proteins (PBPs), inhibiting transpeptidation, the last step of bacterial cell wall synthesis. Additional structures or substituent groups added to the β-lactam ring determine whether the agent is classified as a penicillin, cephem, carbapenem, or monobactam (1-3).

They are the most commonly prescribed agent to fight against bacterial infections. The predominant mechanisms of Resistance to these antimicrobial agents is through the production of β-lactamases (1-3).β-lactamase, the major resistance determinant for β-lactam antibiotics in Gram-negative bacteria, is ancient enzymes whose origins can be traced back millions of years ago(1-2).

These well-studied and characterized enzymes, now numbering nearly 2,800 unique proteins, originally emerged from environmental sources, most likely to defend a producing bacterium from attack by naturally occurring β-lactams. Their ancestors were probably penicillin-binding proteins that share sequence homology with β-lactamases possessing an active-site serine (1-2).

The introduction of the third-generation cephalosporins in the early 1980s was heralded as a major discovery in the fight against β-lactamase-mediated bacterial resistance to antibiotics (1,2). Gram negative bacteria such as *Enterobacteriaceae*, *P. aeruginosa* and *Acinetobacter* species are the most important causes of serious hospital and community acquired bacterial infections in humans(2)

The most problematic β-lactamaseenzymes in the clinical community include extended-spectrum β-lactamase (ESBLs) and the serine and metallocarbapenemases(1-4).Extended-spectrum β-lactamase (ESBLs) canhydrolyze and cause resistance to various types of beta-lactam antibiotics, including the oxyimino-cephalosporins and monobactams, but not the cephamycins and carbapenems (2).

The two classificationsschemesfor β-lactamases in use todayare molecular and functional classification schemes. The molecular classification is basedon the amino acid sequence and divides β-lactamases into class A, C, and D enzymes which utilize serinefor β-lactam hydrolysis and class B metalloenzymes which require divalent zinc ions for substratehydrolysis. The functional classification scheme takes into account substrate and inhibitor profiles in attempt to group the enzymes in ways that can be correlated with their phenotype in clinical isolates(3,4).

The updated system includes group1 (class C) cephalosporinases; group 2 (classes A and D) broa d-spectrum, inhibitor-resistant, and extended-spectrum β-lactamases andserine carbapenemase; and group 3 Metalloβ-lactamases(3,4). ESBLs are derived from genes for the narrow-spectrum beta-lactamases (TEM-1, TEM-2, or SHV-1) by mutations that alter the amino acid configuration around the enzyme active site. Organisms that produce ESBLs remain important causes of the failure of therapy with cephalosporins and have serious infection control consequences(2).

Carbapenemases are the most versatile family of β-lactamase and recognize almost all hydrolyza ble β-lactams, and most are resilient against inhibition by all available β-lactamase inhibitors (5–7).*Klebsiella pneumonia* carbapenemases (KPC) hydrolyze penicillins, all cephalosporins, mono bactams, carbapenems, and β-lactamase inhibitors. Metallo β-lactamases usually exhibit resistan ce to penicillins, cephalosporins, carbapenems, and the clinically available β-lactamase inhibitors but inhibited by monobactams(8–12).

1.2. **Statement of the Problem**

ESBL-producing *Enterobacteriaceae*(ESBL-E) and carbapenem-resistant *Enterobacteriaceae* (CRE) pose a major clinical problem worldwide (13). According to the 2017 WHO priority path ogens list for Research &Development of new antibiotics, carbapenem-resistant *Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacteriaceae*, ESBL producing *Enterobacteria ceae* were classified as priority 1 or critical pathogens(14).

The economic impact related to antimicrobial resistance was expected to cost over $105 billion annually worldwide and the largest relative economic impact was projected to be suffered by Africa with a drop in GDP of US $2895 billion in 2050, representing 20 % of the region's total economic output(15).

Many deaths have occurred as a result of this in Europe, and the European Centre for Disease Prevention and Control (ECDC) had estimated that 25,000 people may die each year from infections related to antimicrobial resistance (16,17). The reported annualcosts of combating resistant bacterial infections are between $21 billion and $34 billion in the USA alone (18).

From European Antimicrobial Resistance Surveillance System (EARS-Net) data collected in 2015, the European Centre for Disease Prevention and Control (ECDC) estimated 670,000 infections with antibiotic-resistant bacteria, and these infections accounted for an estimated 33,000 attributable deaths(19).

The United States Centers for Disease Control and Prevention (CDC) reported antibiotic-resistant bacteria and fungi cause more than 2.8 million infections and 35,000 deaths in the United States each year. That means someone in the United States gets an antibiotic-resistant infection every eleven seconds and every fifteen minutes someone dies (20). CDC listed Carbapenem-resistant *Acinetobacter and Enterobacteriaceae* (CRE) as Urgent Threats and Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* and Multidrug-resistant *Pseudomonas aeruginosa (P. aeruginosa) as* Serious Threats(20).

In the U.S the revised estimate shows that more than 2.6 million antibiotic-resistant infections and nearly 44,000 deaths occurred each year when the 2013 report was published. When

compared to the previous estimate, the updated 2013 report estimate the number of deaths caused by antibiotic resistance each year is nearly two-times higher(20).

Due to the lack of available therapeutic options, the Centers for Disease Control and Prevention (CDC) listed carbapenem-resistant members of the family Enterobacteriaceae as an “immediate public health threat that requires urgent and aggressive action” Centers for Disease Control and P revention(20).

Patients with bloodstream infections caused by ESBL producing *Enterobacteriaceae* are nearly 5 7% more likely to die than those with bloodstream infections caused by a non-ESBL-producing strain. An estimated 140,000 healthcare-associated *Enterobacteriaceae* infections occur in theUn ited States each year (21).

CDC estimates that bloodstream infections caused by ESBLcontaining *Enterobacteriaceae* result in upwards of $40,000 in excess hospital charges per occurrence. Approximately 26,000 infectio ns and 1,700 deaths are attributable to ESBLs (20). According to a recent World Health Organiza tion report, ESBL producing *Enterobacteriaceae* are a critical human health concern (21).

New Estimate of Annual Deaths Caused by Treatment-Resistant Infections showing that as many as 162,044 people die from multi-drug resistant infections annually in the United States. The estimates, nearly seven times higher than 2013 estimates from the U.S. Centers for Disease Control and Prevention, are based on 2010 mortality data(22).

High mortality rates, ranging from 30% to 75%, have been reported for patients with severe CRE infections (23). Mortality above 50% has been reported in patients with CRE bloodstream infections (24), and a study has shown excess mortality of 27% in patients with pneumonia or bloodstream infections caused by carbapenem-resistant *K. pneumoniae*(25).

Globally, about 700,000 people die due to AMR related illnesses every year. It is estimated that by 2050 these deaths will reach10 million, costing the world US$100 trillion(26).The study conducted at Tikur Anbessa Specialized hospital from August 2012 to October2013, reported fivefold increase in mortality rate among patients with positive blood culture results and the death of 11 patients with *Enterobacteriaceae* resistant to third generation cephalosporins(27).

In Ethiopia, few studies have compared ESBL detection methods and few studies were undertaken to determine the prevalence of carbapenem-resistant and carbapenemase-producing gram-negative bacilli. Hence, this study was aimed to compare four phenotypic assays used for the detection of Extended-spectrum β-lactamase producing *Enterobacteriaceae* and to determine the prevalence of carbapenem-resistant and carbapenemase-producing gram-negative bacilli at Ethiopian Public health Institute.

1.3. Significance of the study

Given the importance of extended-spectrum cephalosporins and carbapenem-resistant gram-negative bacilli, the results of this study will;

- Be used as baseline data for further antimicrobial resistance studies
- Provide information for microbiologists to use the best assays of ESBL screening and confirmatory methods
- Provides information for Physicians/clinicians on which antibiotics to use for treating patients infected by carbapenemase producing gram negative bacilli
- Provides information for Physicians/clinicians to initiate empirical treatment in case culture and antimicrobial susceptibility is delayed and not requested
- Provides baseline data for policy makers on the current antibiotic resistance pattern of gram-negative bacilli
- Be used by health facility Antibiotic stewardship and Infection prevention committee
- Be used for Infection control and epidemiological purposes

2. **Literature review**

Antibiotic Resistance happens when germs (bacteria and fungi) defeat the drugs designed to kill them. Any antibiotic used to treat infection of people, animals, or cropscan lead to resistance. Resistantgerms are a One Health problem meaning that they can spread between people, animals, and theenvironment (e.g., water, soil) (20).

Penicillin is the first β-lactam antibiotics and, was discovered in 1928 by Alexander Fleming. There has been a discovery of resistance together with the discovery of new antibiotics. Germs will always search for mechanisms to survive and resist new drugs. More and more, germs are sharing their resistance, making it harder for us to keep pace with it (20).

β-lactams are antimicrobial classes for which resistance has become a major problem.The production of β-lactamases are common mechanisms of resistance to β-lactam antibiotics. ESBL and carbapenemases are the common mechanisms by which gram-negative bacteria develop resistance to β-lactam antibioticsThere are two classifications methods for β-lactamases in use today are molecular and functional classification schemes. The molecular classification divides β-lactamases into classes A, B, C, and D enzymes. Carbapenemases are molecular class A, B, and D (1-4,28).

A variety of class A carbapenemases has been described; the most important one is KPC. Ambler class a ß-lactamases is, at least partially, inhibited by ß-lactamase inhibitors such as clavulanic acid and tazobactam, whereas Metallo-β-lactamases are inhibited by divalent cation chelators such as EDTA. Class B Metallo-β-lactamases (MBLs) are mostly of the Verona integron-encoded Metallo-β-lactamase (VIM) and IMP types and, more recently, of the New Delhi Metallo-β-lactamase-1 (NDM-1) type(28).

The first report of plasmid-encoded β-lactamases capable of hydrolyzing the extended-spectrum cephalosporins was published in 1983(29).The first enzyme with β-lactamase activity reported in the literature in 1940 was from *Bacillus coli*, now assumed to be the class C, AmpC chromosomal cephalosporinase from *Escherichia coli*(30).

The first plasmid-mediated β-lactamase in gram-negative bacteria TEM 1 was described in the early 1960s(31).Among the many ESBLs described in a variety of pathogens, CTX-M, TEM, and SHV types are the most important ones. The TEM-1 enzyme was originally found in a single strain of *E. coli* isolated from a blood culture from a patient named Temoniera in Greece, hence the

designation TEM(32). Because this β-lactamase was plasmid-borne, it soon spread to other mem bers of the Enterobacteriaceae family, *H.influenzae, Neisseria gonorrhoeae*, and *Pseudomonas aeruginosa*(33).

SHV-1is another β-lactamase commonly found in Klebsiella and *E.coli*. Over the years, the use of newer β-lactam antibiotics has enabled the selection of new variants of β-lactamases. In the early 1980s, the third-generation, or oxyimino, cephalosporins were introduced into clinical practice in response to the increasing prevalence and spread of the β-lactamases (34).

Resistance to these extended-spectrum cephalosporins emerged quickly, and the first report of an SHV-2 enzyme which was capable of hydrolyzing these antibiotics was published as early as 19 83 in Germany *from Klebsiella ozaenae* isolates(35).Because the enzyme was active against the expanded-spectrum β-lactam antibiotics, this and subsequent enzymes with the same ability were designated “extended-spectrum β-lactamases” (ESBLs)(33).

The second-largest group of ESBLs is the CTX-M enzymes. Most of these subgroups have evolved as a result of the chromosomal β-lactamase genes escaping from *Kluvera* spp., an enterobacterial genus of little clinical importance. Having migrated to mobile DNA, the CTX-M β-lactamases may evolve further. *Enterobacteriaceae* (mostly *Escherichia coli*) producing the CTX-M enzymes have been identified, predominantly from the community, as a cause of urinary tract infections(35). Various reports suggest that the CTX-M-type ESBLs may now actually be the most frequent ESBL type worldwide(36).

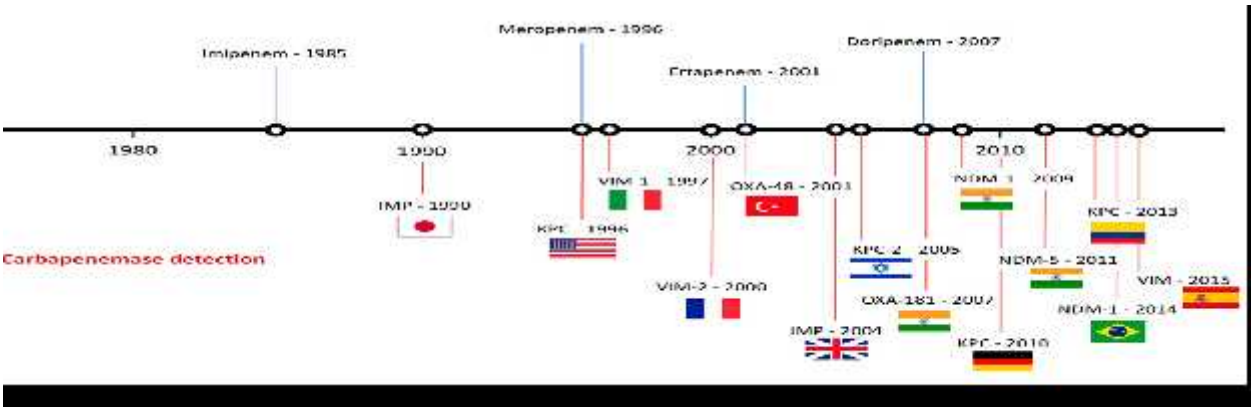


Figure 1:Carbapenem antibiotics and carbapenemase enzymes historical timeline.

IMP-1 was first isolated from *P. aeruginosa* isolates in Japan in 1990 (37). *Verona* integron-encoded MBL was first detected in a carbapenem-resistant *P.aeruginosa* clinical isolate from an Italian inpatient admitted at the Intensive Care Unit Department of the Verona University Hospital, Italy in February 1997(38).

The first strain recorded with a KPC enzyme, in 1996,was a *K pneumoniae*isolate collected in a North Carolinahospital and submitted to the Centers for Disease Controland Prevention (CDC) through Project Intensive CareAntimicrobial Resistance Epidemiology (ICARE)(11).

NDM-1was first reported from *K.pneumoniae* and *E. coli* strains isolated from a Swedish patient of Indian origin traveled to New Delhi, India, in 2006 acquiring UTI caused by a carbapenem-res istant *K.pneumoniae* (39). In Africa, Morocco, Kenya, and South Africa have reported NDM-1 as the most dominant carbapenemase gene(40,41).

However, South Africa was the first to have reported a KPC-2-positive organism in 2012 (42). The study from Ghana during 2016 detected genes blaNDM-1genes in *A.baumannii,* blaVIM-1 in *Pseudomonas* species and blaOXA-48 in *Klebsiella pneumonia* (43).

A cross-sectional survey conducted at four hospitals in Harar, Ethiopia revealed 19/57 (33.3%) of ESBL producing *Klebsiella* isolates(44). The study conducted at EPHI from January 1 to May 30, 2017and from December 2017 to June 2018 revealed the magnitude of ESBLs-E57.7 %(246/426) and67% respectively(45,46).

The study conducted from January to March 2014among children suspected of septicemia and urinary tract infections at Tikur Anbessa Specialized Hospital revealed the overall prevalence of

ESBL and carbapenemase-producing *Enterobacteriaceae* 78.57% (n=22/28) and 12.12% (4/33) r espectively(47). Another study done during December 2012 on the gastrointestinal colonization r ate at Tikur Anbessa Specialized Hospital by ESBL producing *Enterobacteriaceae* (ESBL-E) revealed an overall prevalence of 52% (48).

The study done at Arsho Advanced Medical Laboratory, Ethiopia on non-fermentative gram-negative bacilli from October 2016 to September 2017 revealed 66/135 (48.9%) overall prevalen ce of ESBL (49). Another study conducted from February to May 2014 among 442 symptomatic UTI suspected patients at the University of Gondar, Ethiopia showed the overall prevalence of ca rbapenemase-producing *Enterobacteriaceae* 5/183(2.73%) (50).

The study conducted from March to October 2014 at JUSH on 112 isolates screened positive for ESBLs by Vitek 2 compact showed, 63.4% (71/112) positive for ESBL encoding genes by Check-MDR array(51).Another study conducted at Jimma University Specialized Hospital (JUSH)from
March to June 2016 on *E. coli* and *K. pneumoniae* from Patients with Community Onset Urinary Tract Infections revealed 17/74 (23.0%) for ESBL production(52).

The study at JUSH from January 2014 and June 2015 first reported blaNDM-1 *A.baumannii* in three clinical isolates from Jimma, Ethiopia(53).The study at AdamaTeaching Hospital, Ethiopia from May 2013 to June 2014 detected 17/68(25%) ESBL from *Enterobacteriaceae* (54).

The study conducted at Asella Teaching Hospital from April 2016 to June 2018 detected blaND M1in one *K. pneumoniae* isolate and *A. baumannii*. The study also detected, ESBL genes such as TEM17 , CTX-M, SHV-6 and CTX-M high prevalence(81%) of ESBL and 7.4% carbapenemase producing bacteria(55).

3. Objectives

3.1. General Objective

To Compare four phenotypic assays for the detection of ESBL producing gram negative bacteria and assess the magnitude of carbapenemase-producing gram-negative bacilli.

3.2. Specific objectives

- Compare four phenotypic assays used for the detection of extended-spectrum β-lactamase from Enterobacteriaceae.
- To assess the magnitude of carbapenem-resistant and Carbapenemase-producing gram-negative bacilli.
- To determine antimicrobial susceptibility patterns of gram-negative bacilli.

4. Study methods and materials

4.1. Study area

The study was conducted at the National Clinical Bacteriology and Mycology Reference Laboratory on clinical samples collected there and referred from different health care settings in Addis Ababa. The samples were referred from the following hospitals, clinics and health center in Addis Ababa:- AaBET Hospital, St. Paul's Hospital Millennium Medical College hospital , Ras Desta Damtew memorial Hospital, Yekatit 12 Hospital , Minilik Hospital, Saint peter Hospital, Tikur Anbessa Specialized Hospital, Armed Hospital, Police Hospital, Tirunesh Beijing Hospital, Zewditu memorial Hospital, Hayat Hospital, Amin General Hospital, Betzeta Hospital, Alert Hospital, Alem Tena Higher clinic, Epharm clinic, Kechene clinic, Saint Lukas clinic, Kirkos Health center, Ringroad clinic, Selam Health center, Tsigereda speciality clinic, Addisu gebeya health center, Addis reay health center, Hidase clinic, Kolfe health center, Connel medium clinic, and Cathedral speciality clinic

It is located in the Ethiopian Public Health Institute under Bacterial, Parasitic, and Zoonotic research directorate. The laboratory routinely receives and performs identification and antimicrobial susceptibility testing after isolating bacterial pathogens from different clinical samples. The laboratory is accredited by the Ethiopian National Accreditation Office (ENAO) since July 2017. The laboratory was awarded by The African Society for Laboratory Medicine (ASLM) with a certificate of recognition for Achieving ISO accreditation and best practice in Laboratory Medicine after going through ASLM SLIPTA audits at their annual conference conducted in Abuja, Nigeria in 2018. It is also graduated by the American Society for Microbiology.

4.2. Study design and Period:

The prospective cross-sectional study wasconducted from June 30, 2019, to May 30, 2020.

4.3. Population:

4.3.1. Source population:

All gram-negative bacterial isolates identified from different clinical specimens at Ethiopian Public Health Institute's National Clinical Bacteriology and Mycology Reference laboratory.

4.3.2. Study Population:

- The study population was all *K. pneumoniae,K. oxytoca, E. coli*, and *P. mirabilis*clinical isolates for comparison of ESBL detection assays.
- Enterobacteriaceae, *Acinetobacter* species, and *P. aeruginosa* known to produce carbapenemase enzymes.

4.4. Inclusion and exclusion criteria

4.4.1. Inclusion criteria:

- All Enterobacteriaceae, *P. aeruginosa*, and *Acinetobacter* species bacillihaving complete patient information.
- *K. pneumoniae,K. oxytoca, E. coli*, and *P. mirabilis* having complete patient information

4.4.2. Exclusioncriteria:

- Similarisolates isolated from the same patient
- Second isolates isolated from the same patient
- Carbapenem-non susceptible *K. pneumoniae,K. oxytoca, E. coli*, and *P. mirabilis*isolates were excluded for ESBL detection assays comparison.

4.5. Study variables

4.5.1. Dependent variables:

- The performance of of each phenotypic assays for ESBL detection
- Magnitude of carbapenem resistant and carbapenemase-producing organisms.

4.5.2. Independent variables:

- Specimen types
- Organisms types
- Hospital wards
- Referring health facility

4.6. Sample size calculation and Sampling method

- The sample size for comparison of ESBL detection assays

According to CLSI EP09-A3(Measurement Procedure Comparison and Bias Estimation Using Patient Sample), At least 40 and preferably 100 patient samples should be used to compare two methods(56). A total of 117 clinical isolates of *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Klebsiella oxytoca* were used.

- The sample size for carbapenemase enzymes

All Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter* species isolated at National Clinical Bacteriology and Mycology Reference Laboratory from June 2019, to May 30, 2020, were included in this study.

4.6.1. Sampling Method

- Aconvenientsampling method was used for comparison of four phenotypic assays of ESBL detection.
- A convenient sample method was used for determining the magnitude of Carbapenem resistant and Carbapenemase producing gram negative bacilli.

4.7. Laboratory analysis

4.7.1. Bacterial identification and Antibiotic susceptibility testing

Bacterial identification: The isolates were identified by conventional biochemical and Vitek 2 compact method as per laboratory SOPs. Citrate agar slant, Triple sugar iron agar slant, Lysine iron agar slant, Urea agar slant, Sulfide indole motility media were used for conventional bioche mical identification method. Vitek 2 GN identification cards were used for Vitek 2 compac syste ms.117 clinical isolates of *E. coli, K. pneumoniae, P. mirabilis, K. oxytoca* were screened for ES BL using four phenotypic assays. About
429 isolates were used for determining the magnitude of carbapenem resistant and carbapenemas e producers.

Antibiotic susceptibility testing: Antibiotic susceptibility patterns of Ampicillin(10µl), Amoxicillin/Clavulanic acid(20/10µl), Ampicillin/Sulbactam(10/10µl), Piperacillin/Tazobactam(100/10µl), Cefazolin(30µl), Cefuroxime(30µl), Ceftazidime(30µl), Ceftriaxone(30µl), Cefotaxime(30µl), Cefepime(30µl), Ertapenem(10µl), Imipenem(10µl), Meropenem(10µl), Amikacin(30µl), Gentamicin(10µl), Tobramycin(10µl), Nalidixic acid(30µl), Ciprofloxacin(5µl), Trimethoprim/Sulfamethoxazole (1.25/23.75µl), Nitrofurantoin(300µl), Chloramphenicol(30µl), Tetracycline(30µl) were determined in vitro by Kirby-Bauer disk diffusion method following Clinical and Laboratory Standards Institute guidelines(56).

- **Screening for ESBL producing isolates**

ESBL-producing gram-negative bacilli were first screened for ESBL production by indicator cephalosporins; cefotaxime (30µg) and Ceftazidime (30µg).Isolates having a zone of inhibition ≤22mm and ≤27 mm for ceftazidime and Cefotaxime respectively, were considered a potential ESBL producer.The suspected isolate was further confirmed by the phenotypic confirmatory test as per the European committee on Antimicrobial Testing (EUCAST, 2017) and the Clinical and Laboratory Standard Institute (CLSI) guidelines(57,58).

4.7.2. **Combined disk method**:

Confirmation of suspected ESBLs producers was done by using Ceftazidime 30µg, Ceftazidime clavulanate (30/10µg), Cefotaxime 30µg, and Cefotaxime-clavulanate (30/10µg) method on Mueller–Hinton agar, as recommended by CLSI guidelines 2020 [57]. The plates were incubated at 37ºC for 16–18 hours and A ≥ 5-mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanatevs the zone diameter of the agent when tested alone was considered ESBL producers (57).

K. pneumonia ATCC®700603 and *E. coli* ATCC®25922 control strains were used for checking the qualityof Ceftazidime 30µg, Ceftazidime clavulanate(30/10µg), Cefotaxime30µg, and Cefotaxime clavulanate (30/10µg)(57).

4.7.3. **Gradient test method**

MIC Test Strip ESBL Cefotaxime (CTX)/Cefotaxime+Clavulanic acid (CTL), Ceftazidime (CAZ)/Ceftazidime+Clavulanic acid (CAL) strips wereused to confirm the presence of clavulanic acid inhibitable ESBL (Extended Spectrum Beta-Lactamase) enzymes in *Escherichia coli*, *Klebsiella pneumoniae,* and *K. oxytoca* and *P.mirabilis.*CTX ≥ 0.5 and CTX/CTL ratio ≥ 8 or CAZ ≥ 1 and CAZ/CAL ratio ≥ 8 or "Phantom" zone or deformation of the CTX, CAZ, or FEP ellipse was considered ESBL positive. CTX < 0.5 or CTX/CTL ratio < 8 and CAZ < 1 or CAZ/CAL ratio < 8 was considered ESBL negative(59).

4.7.4. **Double-disk synergy test (DDST)**

Disks containing cephalosporins (cefotaxime and ceftazidime) were applied to plates next to adisk with clavulanic acid (amoxicillin-clavulanic acid). A positive result was indicated when the inhibition zones around any of the cephalosporin disks are augmented or there is a 'keyhole' in thedirection of the disk containing clavulanic acid. The distance between the disks was 20mm from center-to-centeris optimal for cephalosporin 30µg disks.However, the distance between the disks wasreduced (15 mm) or expanded (30 mm) for strains with very high or low levels of resistance, respectively(58).

4.7.5. **Vitek 2 ESBL testing**

VITEK cards for susceptibility testing (AST-**GN86**) were used.The results were interpreted by using software version 7.0 and an advanced expert system (AES) designed to analyze the results generated by the VITEK 2 system(60).

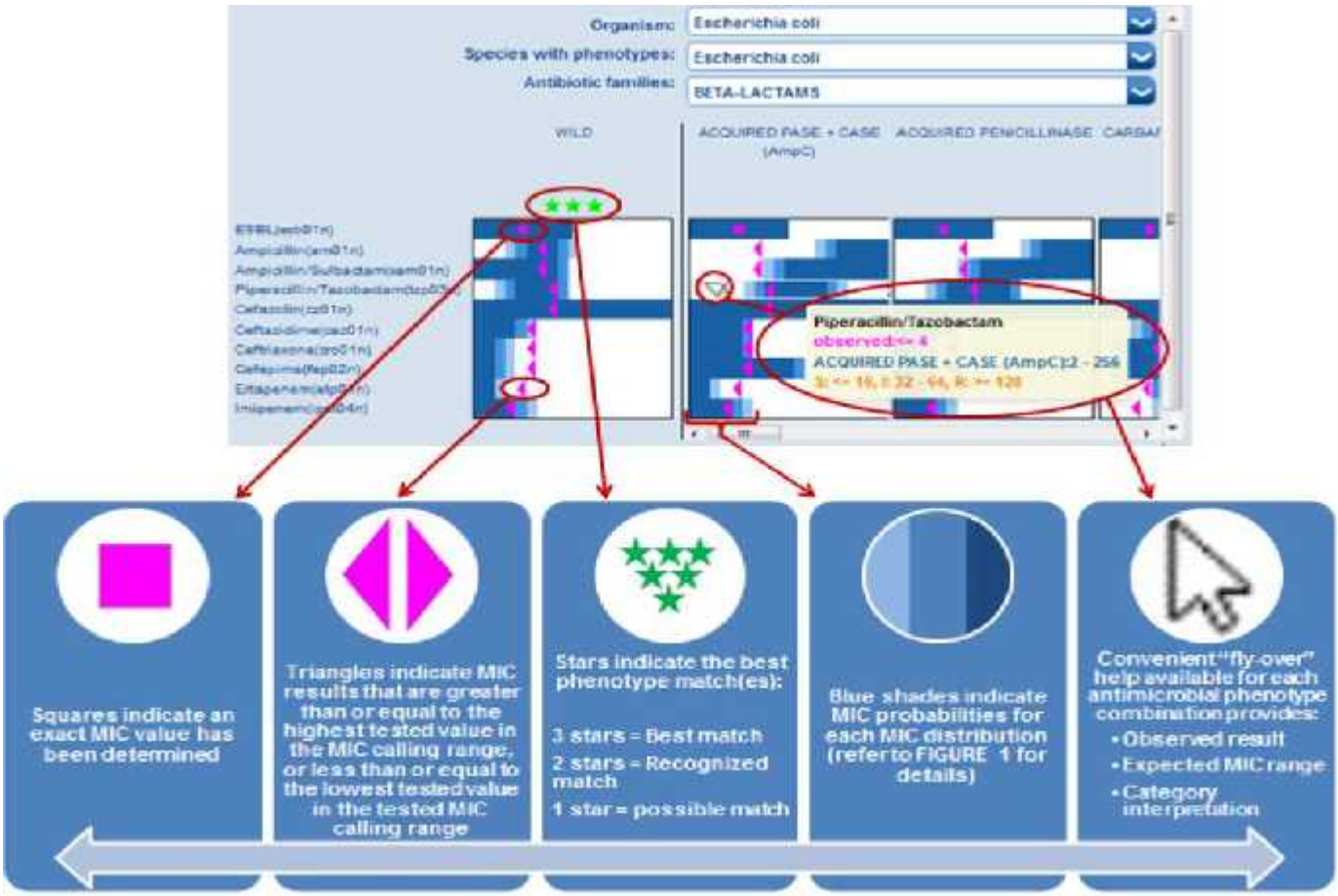


Figure 2 VITEK 2 technology advanced expert system

VITEK 2 technology with the ADVANCED EXPERT SYSTEM offers：

- Knowledgebase developed from >100,000 references
- >2,000 described phenotypes
- >20,000 MIC distributions
- >100 resistance mechanisms detected
- >99 organisms
- On average, provides a resulting range of five to seven MIC doubling dilutions per antibiotic
- Extended MIC range to enable low-level resistance detection
- Resistance-oriented results that highlight unusual phenotypes
- Deduced antibiotic results to meet formulary requirements
- Checks every result every time
- The Advanced Expert System™ (AES) - provide rapid, accurate "fingerprint" recognition of bacterial

(I) results are fully consistent with identification; (ii) results are not fully consistent with identification; therefore, modify the susceptibility results as suggested by the AES, or retest isolate; (iii) results are not fully consistent; therefore, modify the susceptibility results, or change the identification as suggested by the AES, or retest isolate; and (iv) results are not fully consistent, and the isolate should be retested(60).

4.7.6. **Detection of carbapenemase**

CLSI (2010) introduced the modified Hodge test for carbapenemase detection, but this method can only be used for the accurate detection of KPC-type carbapenemase in *Enterobacteriaceae*(61).CLSI (2012) recommended the Carba NP test method for the detection of carbapenemase in gram-negative bacilli; however, the preparation of the reagents required for this test is complicated and the solutions cannot be stored for extended periods, limiting its clinical application(62).

In 2015 a new detection method, carbapenem inactivation method (CIM), which is easy to operate and highly sensitive in the detection of carbapenemase was designed(63). In 2017, based on the CIM method, CLSI recommended the modified carbapenem inactivation method (mCIM).This method is effective at detecting a variety of carbapenemases(64).However; it is a relatively complex method and can only be used to detect carbapenemase in Enterobacteriaceae and *P. aeruginosa*(57). In 2018, based on the mCIM, a simplified carbapenem inactivation method (sCIM) was designed for simple and accurate detection of carbapenemase in gram-negative bacilli(65).

4.7.7. **Modified Carbapenem Inactivation method.**

In the mCIM, 1 mL loop full of***Enterobacteriaceae***or 10 mL loop full of *P. aeruginosa* from blood agar plates was emulsified in 2 mL trypticase soy broth (TSB). A meropenem disk was then be immersed in the suspension and incubated for a minimum of 4 h at 35ºC. A 0.5 McFarland suspension of *E. coli*ATCC 25922 was prepared in saline using the direct colony suspension method. A MuellerHinton agar (MHA) plate was inoculated with E. coli ATCC 25922 using the routine disk diffusion procedure. The meropenem disk was removed from the TSB and placed on an MHA plate previously inoculated with the *E. coli*ATCC 25922 indicator strains. Plates were incubated at 35ºC in ambient air for 18-24 h. An inhibition zone diameter of

6-15 mm or colonies within a 16–18 mm zone was considered to be a positive result, and a zone of inhibition ≥19 mm wasconsidered to be a negative result (57).

4.7.8. Simplified Carbapenem Inactivation Method

The sCIM is based on the mCIM with an improvement of experimental procedures. Instead of incubating the antibiotic disk in the organism culture media for 4 hours as in the mCIM, the organism to be tested wassmeared directly onto an antibioticdisk in the sCIM. To perform the sCIM, for *Acinetobacter species*, a 0.5 McFarland standardsuspension (using direct colony suspension method) of *E. coli*ATCC 25922 was diluted 1:10 in saline and inoculated onto theMHA plate, following the routine disk diffusion procedure. Plateswere allowed to dry for 3–10 min(65).

Then, 1–3 overnight colonies of the test organisms grown on blood agar was smeared ontoone side of an imipenem disk (10µg); immediately afterward, the side of the disk having bacteria was placed on the MHA plate previouslyinoculated with *E. coli* ATCC 25922. An imipenem disk placed on an MHA plate was used as the control(65).

All plates were incubated at 35C for 16–18 h in ambient air. Bacterial strainsthat produced carbapenemase can hydrolyze imipenem; hence the susceptible indicator strain grew unchecked.

In contrast, the zone of inhibition around the disk shows a diameter of 6–20 mm, or the satellite growth of colonies of *E. coli*ATCC 25922 around the disk with a zone diameter ≤22 mm, indicating that the isolate is capable of producingcarbapenemase; a zone of inhibition ≥26 mm was considered to be a negative result; a zone of inhibition of 23–25 mm was considered to be a carbapenemase indeterminate result(65).

4.7.9. **Confirmation of *Klebsiella pneumoniae* carbapenemase (KPC)**

The Modified carbapenem inactivation method positive Enterobacteriaceae and *P. aeruginosa* and Simplified Carbapenem Inactivation Method positive *A. baumannii* detected will be further screened for *Klebsiella pneumoniae* Carbapenemase (KPC). MIC Test Strip KPC strips consisting of Meropenem (MRP)/Meropenem+Phenylboronic acid (MBO) was used to detect *Klebsiella pneumoniae* Carbapenemase (KPC) producing gram-negative(66).

Well, isolated colonies from an overnight blood agar plate were Suspend into saline to achieve a 0.5 McFarland standard turbidity (1 Mc-Farland if mucoid) to obtain a confluent lawn of growth after incubation.The strip was applied to the agar surface with the scale facing upwards and the code of the strip to the outside of the plate. The agar plates were incubated in an inverted position at 35 ± 2°C for 16-20 hours in the ambient atmosphere. The incubation time was Extend up to 48 hours in case of slow-growing Gram-negative non-fermenters(66)

Result Interpretation: The ratio of MRP/MBO of ≥8 or ≥3 log2 dilutions were interpreted as KPC producer. The phantom zone or deformation of the ellipse was interpreted as positive for KPC regardless of the MRP/MBO ratio(66).

Quality control recommendations: *E.coli* ATCC® 25922 and *K. pneumoniae* ATCC® BAA-1705 (intrinsic KPC production) were used to check the quality of KPC strips(66).

4.7.10. **Detection of Metallo β-lactamase.**

The Modified carbapenem inactivation method positive Enterobacteriaceae and *P. aeruginosa* and Simplified Carbapenem Inactivation Methodpositive *A. baumannii* detected was further screened for Class B Metallo-carbapenemases (MBLs), which are characterized by inhibition by metal chelators, EDTA. A ≥ 5-mm increase in zone diameter for eCIM vs. zone diameter for mCIM was considered Metallo-carbapenemase producer. A≤4mm increase in zone diameter for the eCIM vs zone diameter of mCIM was considered Metallo-carbapenemasenegative(57).

Quality control recommendation: *K. pneumoniae* ATCC® BAA-1705™ *E.coli* ATCC® 25922™ was used to check the quality of meropenem with EDTA(57).

4.8. Data Quality Assurance

The quality of culture media, antibiotic disks, and gradient strip antibiotics were checked as per CLSI, EUCAST guideline, Laboratory SOPs, and manufacturer's instructions.

4.9. Data analysis and interpretation

The data was being entered, cleaned, and analyzed using SPSS version 23 and WHOnet 2019 version.Because the main goal of ESBL detection is to reach high sensitivity, that means to detect the highestnumber of ESBL-positive strains, we used Ceftazidime/Ceftazidime with clavulanic

acid as reference method. Sensitivity and specificity of each phenotypic test were computed by u sing Ceftazidime/Ceftazidime with clavulanic acid method as the gold standard. Tables and figures wereused for data presentation.

4.10. Quality control

Klebsiella pneumonia ATCC 700603 and *Escherichia coli* ATCC 25922 were used as ESBL positive and negative controls, respectively.

4.11. Operational definitions

- **American Type Culture Collection:**Top quality bacterial strains used for assuring the quality of culture media, antibiotic disks.
- **β-lactam antibiotics**: β-lactam antimicrobial agents that shares a common, central, four-member β-lactam ring.
- **Carbapenemase**: Carbapenemases are β-lactamases that hydrolyze penicillins, in most cases cephalosporins, and to various degrees carbapenems and monobactams.
- **Combined disk method:** Disks containing cephalosporin alone (cefotaxime, ceftazidime, and cefepime) and in combination with clavulanic acid are applied.
- **ESBL**: ESBLs are enzymes that hydrolyze most penicillins and cephalosporins, including oxyimino-β-lactam compounds.

- **Minimal inhibitory concentration (MIC)**: the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in agar or broth dilution susceptibility test.
- **Modified carbapenem inactivation method (mCIM)**: phenotypic micro tube assay for detecting carbapenemase production in which the ability of an isolate to degrade meropenem in a 10-µg disk is assessed.
- **Metallo β-lactamase**: Enzymes that confer resistance to all β-lactams except monobactams
- **Carbapenem non susceptible isolates:** Isolates that are resistant or intermediate to carbapenem antibiotics
- **Multidrug Resistance:** when a bacterium is simultaneously non susceptible to three or more drugs belonging to different classes of antibiotics.

4.12. Ethical considerations

The study was conducted after ethical clearance was obtained from department research and ethical review committee of the department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University. Official permission from Ethiopian Public Health Institute was obtained. All results were kept confidential; the patients name and other personal identifier were not used, rather the sample identification number automatically generated by Polytech was used.

4.13. Dissemination of the Results

The result of this study was submitted to the Addis Ababa University department of Medical Laboratory Sciences. The result will be submitted to Ethiopian Public Health Institutes and will also be submitted to peer reviewed journal for publication.

5. Results

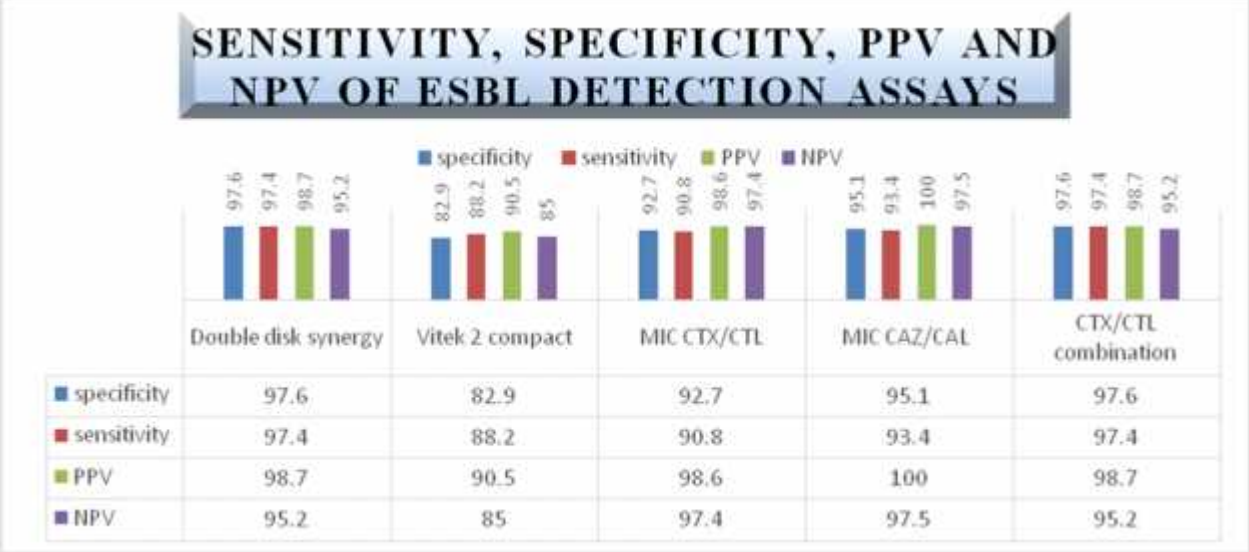
5.1. ESBL phenotypic assays comparison results

One hundred seventy clinical isolates of *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis,* and *Klebsiella oxytoca* collected at Ethiopian Public Health Institute's National Clinical Bacteriology and Mycology Reference Laboratory during the study time was used. 73.5% (86/117) from urine, 10.3% (12/117) from blood, 4.3% (5/117) from pus, 2.6% (3/117) from sputum, 1.7% (2) from middle Ear, 0.9% (1) from tissue, Broncho-alveolar Lavage, pleural fluid and CSF were selected.

Out of 117 bacterial isolates, 54.7% (64/117) were*E. coli*, 34.2% (40/117) were *K. Pneumoniae*, 6 % (7/117) were *P. mirabilis,* and 5.1% (6/117) were *K. Oxytoca (*Table 2*).*Both manual and Vitek 2 compact system identification methods were used for the identification of bacterial isolates.

5.2. ESBL results by different phenotypic assays.

Combination disk method using Disks containing the cephalosporin alone (cefotaxime, ceftazidime,) and in combination with clavulanic acid were used as Reference method as per CLSI 2020 M100.The diagnostic capacity of each phenotypic method was evaluated by analyzing sensitivity,specificity, and positive and negative predictive values.



	Double disk synergy	Vitek 2 compact	MIC CTX/CTL	MIC CAZ/CAL	CTX/CTL combination
specificity	97.6	82.9	92.7	95.1	97.6
sensitivity	97.4	88.2	90.8	93.4	97.4
PPV	98.7	90.5	98.6	100	98.7
NPV	95.2	85	97.4	97.5	95.2

Table 1: Sensitivity, Specificity, PPV, and NPV of the four ESBL of detection assays

5.2.1.Ceftazidime/Ceftazidime with clavulanic acid combination disk method

Among 117 clinical isolates of *K. pneumoniae*, *E. coli*, *P. mirabilis*, and *K. oxytoca* used, 65% (76/117) were positive by Ceftazidime/Ceftazidime with Clavulanic acid combination disk and the remaining 35% (41/117) were negative (Table 1and 3).

- **5.2.2.Cefotaxime/ Cefotaxime with clavulanic acid combination disk method**

Among 117 clinicals used, 64.1% (75/117) were positive by Cefotaxime/Cefotaxime with Clavul anic acid combination disk and the remaining 35.9% (41/117) were negative (Table 1and 3).

- **5.2.3.Double Disk Synergy method**

Among 117 clinical isolates of *K. pneumoniae*, *E. coli*, *P. mirabilis*, and *K. oxytoca* used, 64.1% (75/117) were positive and the remaining 35.9% (42/117) were negative by Double Disk Synergy method (Table 1 and 3).

- **5.2.4.Vitek 2 Compact system**

Among 117 clinical isolates used, 63.2% (74/117) were positive, 33.4% (39/117) were negative, and 3.4% (4/117) werenon-determinable by Vitek 2 compact (Table 1 and 4).

- **5.2.5.MIC test strips ceftazidime/Ceftazidime with clavulanic acid**

Among 117 clinical isolates used, 60.7% (71/117) were positive, 34.2% (40/117) were negative, and 5.1% (6/117) were nondeterminable by MIC test strips ceftazidime/Ceftazidime with clavulanic acid (Table 1and 4).

- **5.2.6.MIC test strips Cefotaxime/ Cefotaxime with clavulanic acid**

Among 117 clinical isolates used, 59.8% (70/117) were positive, 33.3%(39/117) were negative, and 6.8%(8/117) were non-determinable by MIC test strips Cefotaxime/ Cefotaxime with clavulanic acid (Table 1 and 4).

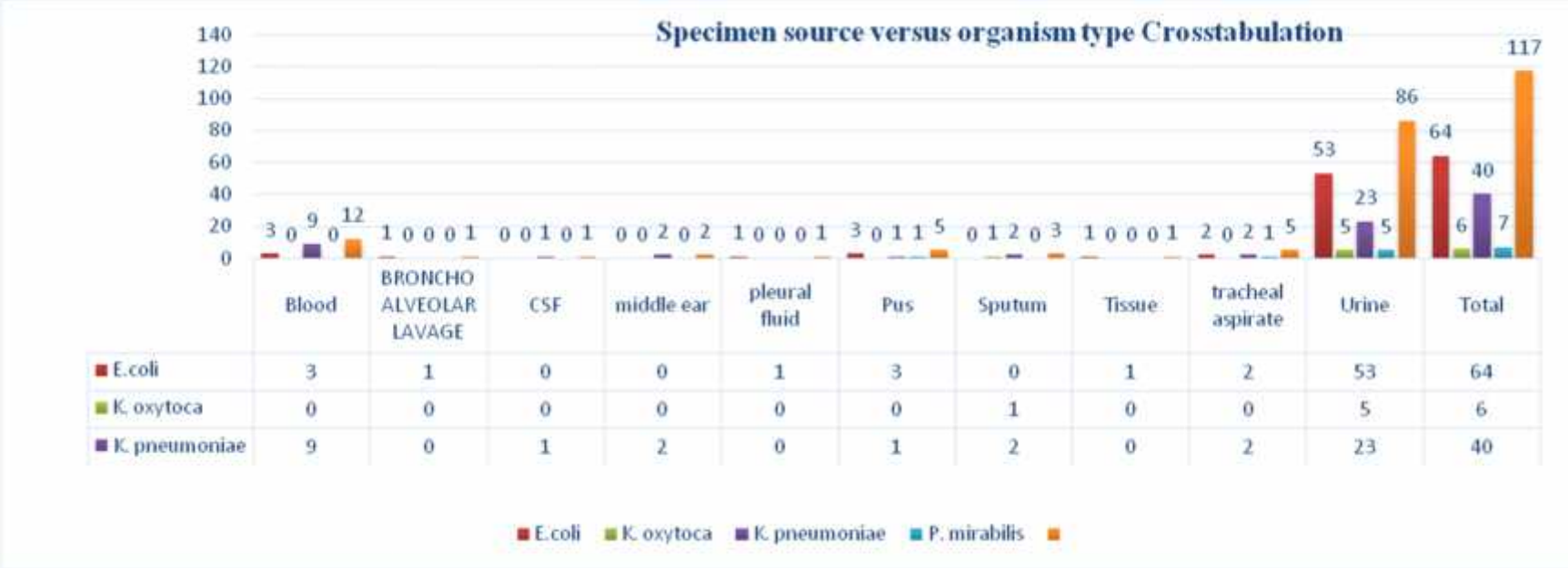


Table 2: Specimen source versusbacterial isolates Crosstabulation

Results	ESBL results by double-disk synergy method		ESBL results by CAZ/CAL combination disk		ESBL results by CTX/CTL combination disk	
	Frequency	Percent%	Frequency	Percent%	Frequency	Percent%
negative	42	35.9	41	35.0	42	35.9
positive	75	64.1	76	65.0	75	64.1
Total	117	100.0	117	100.0	117	100.0

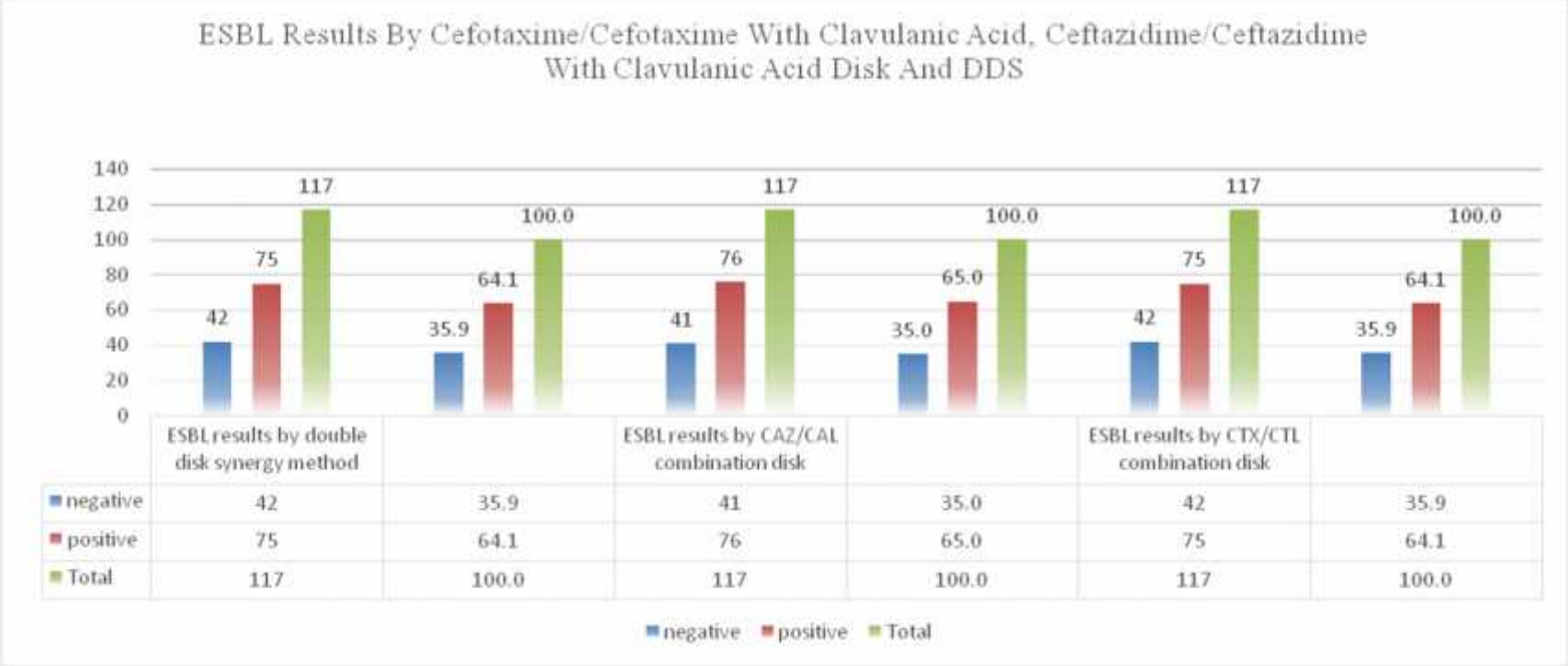


Table 3: ESBL Results using Combination and DDS disk method.

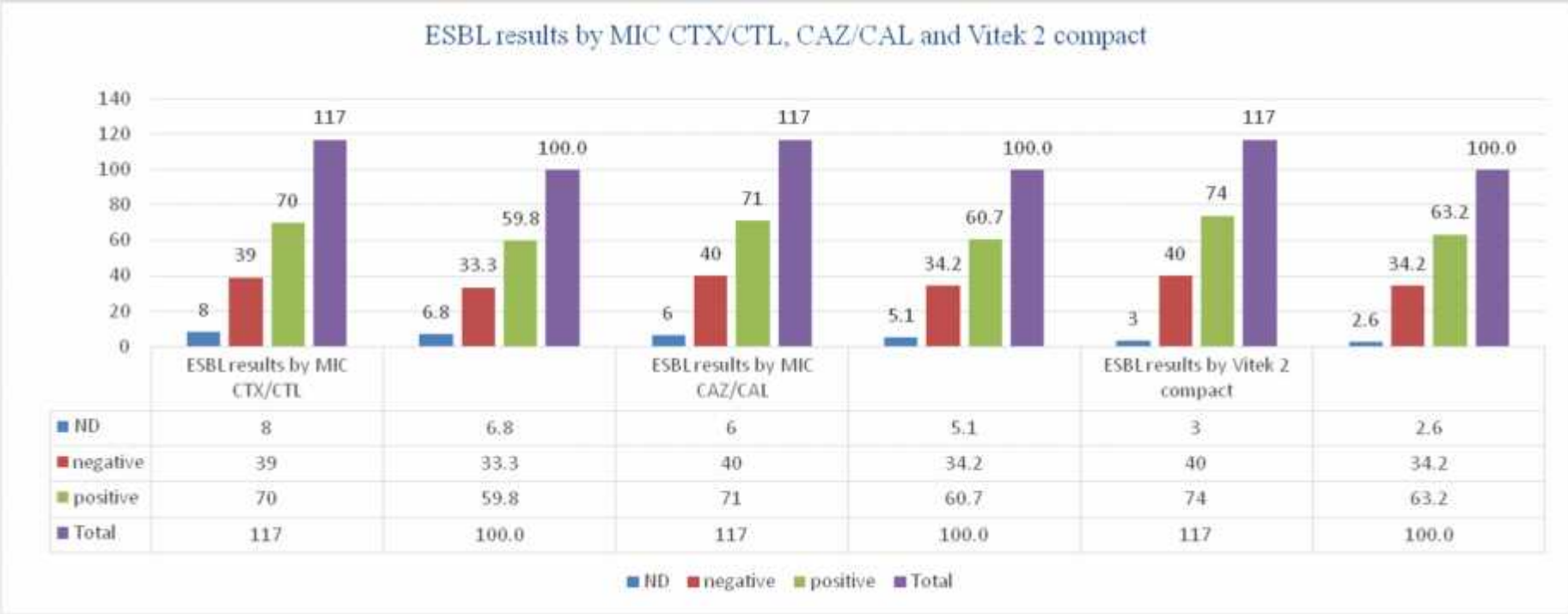


Table 4: ESBL results by MIC CTX/CTL, CAZ/CAL and Vitek 2 compact

A. Vitek 2 compact ESBL results

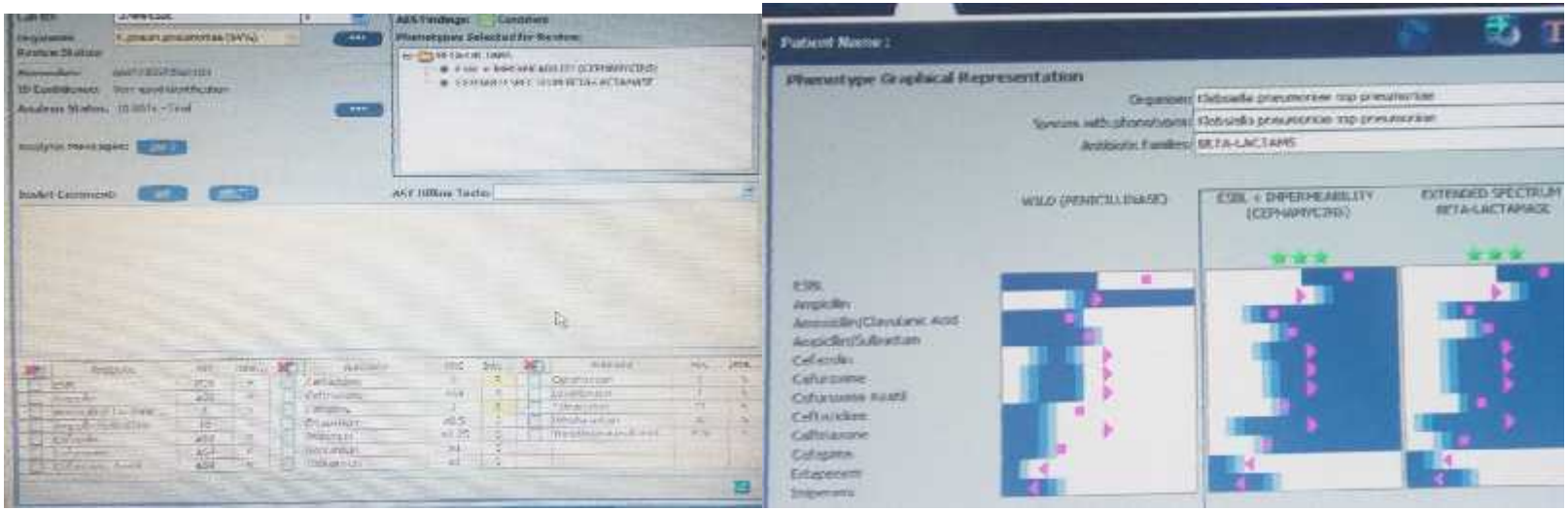


Figure 3: *Klebsiella pneumoniae* positive for ESBL Vitek 2 compact result.

Figure 4: *Klebsiella oxytoca (left)and klebsiella pneumoniae(right)* positive for ESBL by DDS, MIC, combination disk on Muller Hinton agar

Figure 5: ESBL positive *P. mirabilis*(left) and *K. pneumoniae* (Middle)and *E. coli(right)*

5.3. The magnitude of carbapenemase-producing gram negative bacilli results

429 clinical isolates of *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter* species were analy zed to determine the magnitude of carbapenemase producing gram negative bacilli. Meropenem(10µg) and Imipenem(10µg) were tested for 378 and 76 isolates respectively (Figure 7). Both Me ropenem(10µg) and Imipenem(10µg) were tested for 25 isolates (Figure 8).

Out of 25 isolates for which both meropenem(10µg) and Imipenem(10µg) were tested, 68% (17/25) were susceptible to both antibiotics (Figure 8). 16% (4/25) were resistant to both antibiotics (Figure 8). One isolate which was susceptible to meropenem was found to be resistant to Imipenem (Figure 8).12.3% (46/378), 11.8% (9/76) isolates were resistant to meropenem(10ug) and Imipenem(10ug) respectively (Figure 7).2.6% (10/378), 5.3% (4/76) isolates were intermediate to meropenem(10ug) and Imipenem(10ug) respectively (Figure 7).

85.1% (322/378) and 82.9% (63/76) isolates were susceptible to meropenem(10ug) and Imipene m(10ug) respectively (Figure 7).15.4% (66/429) isolates were non-susceptible to either of meropenem or Imipenem (Figure 7).Isolates that werenon-susceptible to either of meropenem or Imipenem, were further screened for the presence of carbapenemase using modified carbapenem inactivation and simplified carbapenem inactivation method.

Out of 66 isolates screened for carbapenemase, 34.8% (23/66) werecarbapenemase enzymes producers (Figure 11).Forty-three out of 66(65.2%) isolates having reduced susceptibility to carbapenems were negative for carbapenemase. Three out of 23 carbapenemase positive organisms were Klebsiella pneumoniae carbapenemase (KPC) producers (Figure 13).

Ten out of twenty-three carbapenemase positive organisms were Metallo-beta-lactamase (MBL) producers (Figure 12). MIC KPC test strips yielded five non-determinable results (Figure 13). 21.74% (5/23) of the 23 carbapenemase positive isolates were found to be Non-MBL and KPC producers. Out of 23 carbapenemase positive isolates, 21.7% (5/23), 8.7% (2/23), 8.7% (2/23),

39.1% (9/23), 17.4% (4/23), 4.4% (1/23) were *Acinetobacter* species, *E. coli*, *K. ozaenae*, K. *pneumoniae*, *P.aeruginosa*, P. *mirabilis*respectively (Figure 11).

Out of 23 carbapenemase positive organisms, 56.5% (13/23), 26.1% (6/23), 8.7% (2/23),8.7% (2/23)were isolated from urine, pus, blood, and tracheal aspirate respectively (Figure 14). Out of 23 carbapenemase positive organisms,82.6% (19/23), 17.4% (4/23) were isolated from the patients admitted to intensive care unit and unknown ward respectively (Figure 15).In this,study the prevalence of carbapenem-resistant and carbapenemase-producing gram-negative bacilli is 11.2% (48/429)and 5.4% (23/429). Three, Seventeen, and Eighty-two out of 429 isolates were resistant to five, four, and three antibiotic classesrespectively. The magnitude of multidrug resistant gram-negative bacilli in this study was 23.77% (102/429).

In the present study, the prevalence of isolates resistant to extended spectrum cephalosporins were, 62.4% (166/266), 67.9% (133/196), 76.9% (113/147), (76% (98/129), Cefepime, Ceftazidime, Ceftriaxone, and Cefotaxime respectively.The prevalence of isolates resistant to Aminoglycosides were, Amikacin 11.2% (30/269), Gentamycin 43.4% (96/221), Tobramycin 38.4% (118/307).

The prevalence of isolates resistant to Nalidixic acid, Ciprofloxacin,and Nitrofurantoin were 80% (20/25), 60.9% (220/361), and 24.8% (39/157) respectively.The prevalence of isolates resistant to Amoxicillin/Clavulanic acid, Piperacillin/Tazobactam, and Ampicillin were 48.8% (78/160), 33.5% (93/278), and 92% (92/100) respectively. The prevalence of isolates resistant to First and second generation cephalosporins, Folate pathway inhibitors, Phenicols were Cefazolin 71.7% (81/113), cefuroxime 73% (108/148), Trimethoprim-Sulfamethoxazole 78.5% (296/377), Chloramphenicol 49.3% (34/69)

Antibiotic name	Breakpoints	Number	%R	%I	%S
Imipenem	20 - 22	76	11.8	5.3	82.9
Meropenem	20 - 22	378	12.3	2.6	85.1

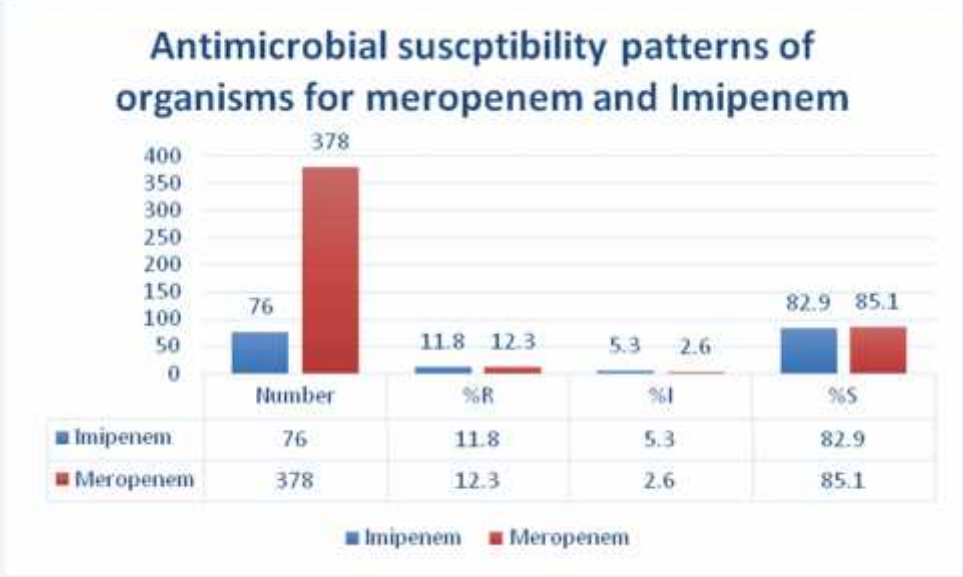


Figure 7: Antimicrobial susceptibility patterns of organisms for meropenem and Imipenem

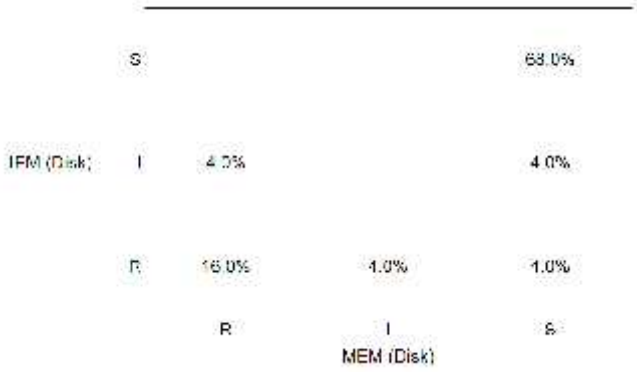


Figure 8:Scatterplot of imipenem vs meropenem antimicrobial susceptibility testing results.

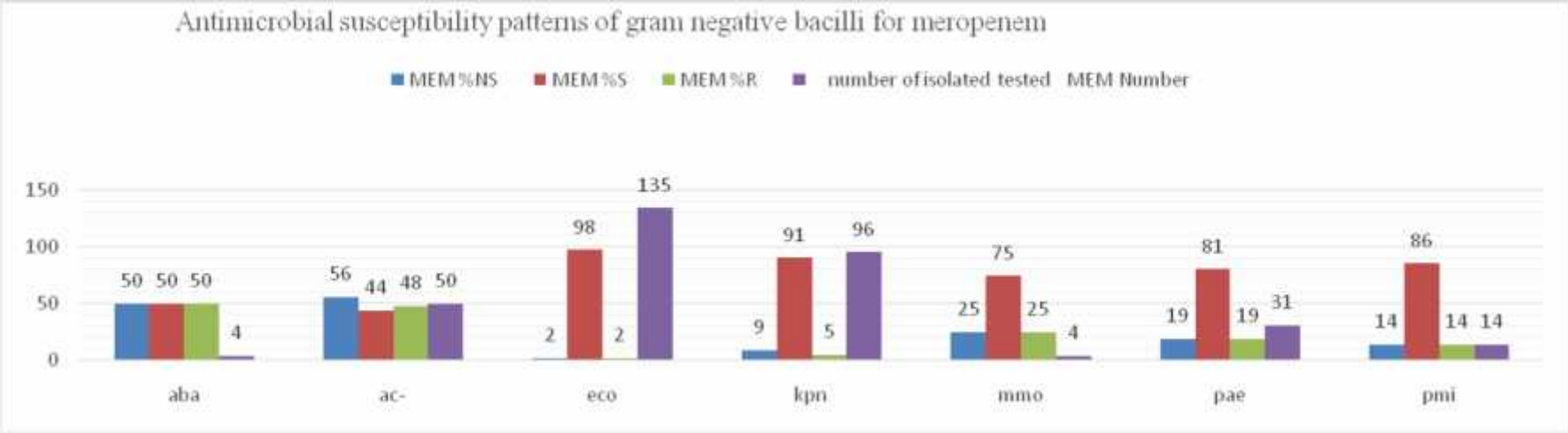


Figure 9:Antimicrobial susceptibility patterns of gram-negative bacilli for meropenem

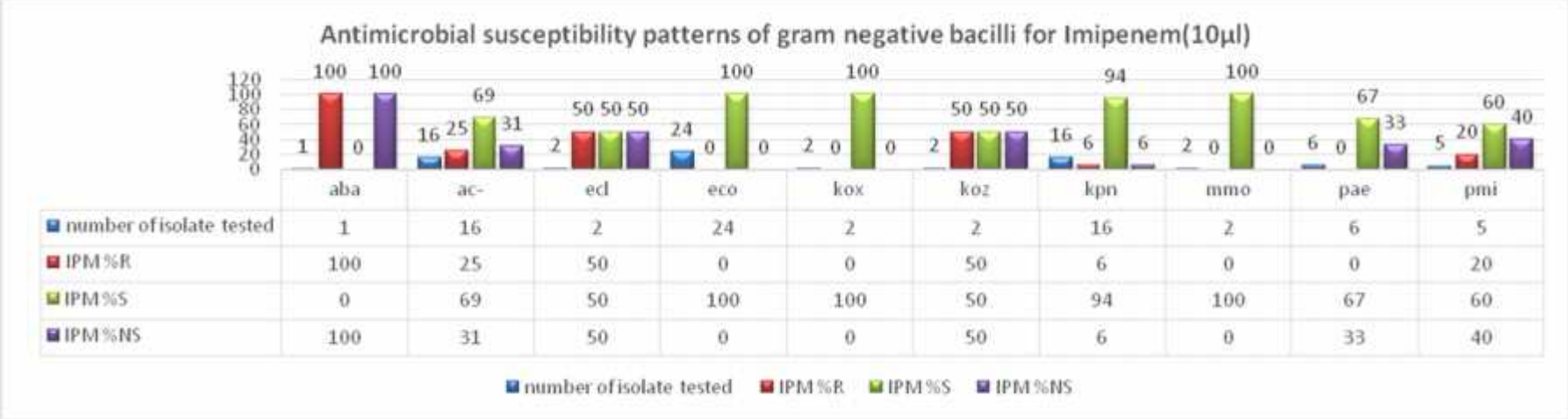


Figure 10: Antimicrobial susceptibility patterns of gram-negative bacilli for Imipenem

code	Organism name	# tested IPM	IPM %R	IPM %S	IPM %NS	# tested MEM	MEM %R	MEM %S	MEM %NS
aba	Acinetobacter baumannii	1	100	0	100	4	50	50	50
ac-	Acinetobacter sp.	16	25	69	31	50	48	44	56
ecl	Enterobacter cloacae	2	50	50	50	11	9	91	9
eco	Escherichia coli	24	0	100	0	135	2	98	2
kox	Klebsiella oxytoca	2	0	100	0	8	0	100	0
koz	Klebsiella pneumoniae ss. ozaenae	2	50	50	50	10	20	70	30
kpn	Klebsiella pneumoniae ss. Pneumonia	16	6	94	6	96	5	91	9
mmo	Morganella morganii ss. morganii	2	0	100	0	4	25	75	25
pae	Pseudomonas aeruginosa	6	0	67	33	31	19	81	19
pmi	Proteus mirabilis	5	20	60	40	14	14	86	14

Table 5:Antimicrobial Susceptibility patterns of gram-negative bacilli to Imipenem and Meropenem

Org code	Organism name	MEM %NS	MEM %S	MEM %R	Number of isolates tested for MEM
eae	Klebsiella aerogenes	0	100	0	2
en-	Enterobacter sp.	50	50	0	2
kl-	Klebsiella sp.	50	50	50	2
pr-	Proteus sp.	0	100	0	2
Pre	Providencia rettgeri	0	100	0	1
Prg	Providencia rustigianii	0	100	0	1
Prv	Providencia sp.	0	100	0	2
Pvu	Proteus vulgaris	0	100	0	3

Table 6: Antimicrobial Susceptibility patterns of gram-negative bacilli to Meropenem

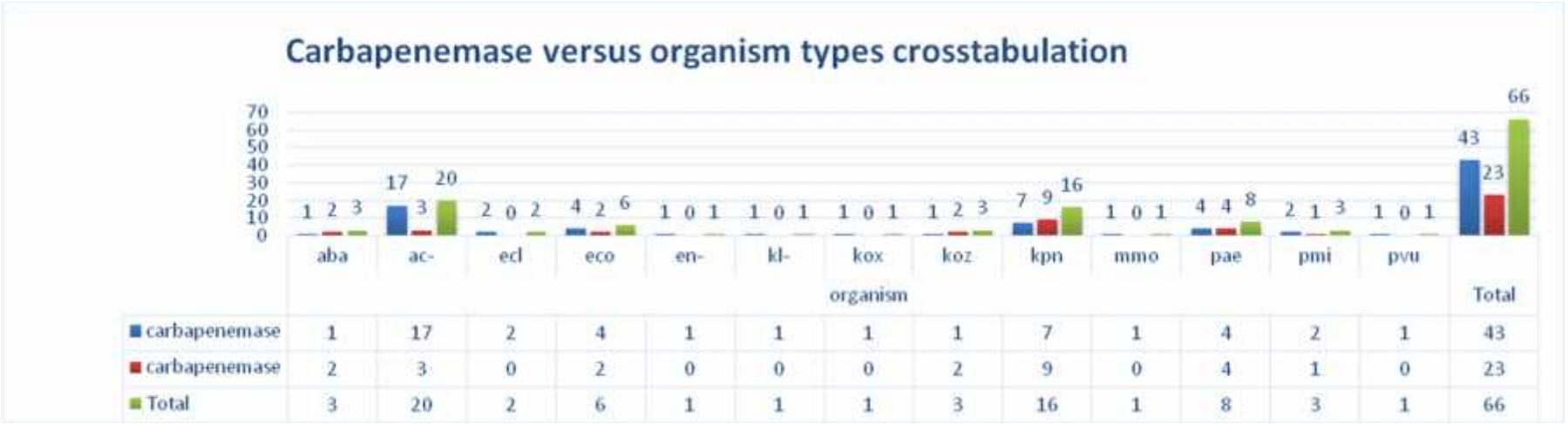


Figure 11: Carbapenemase versus organism types crosstabulation

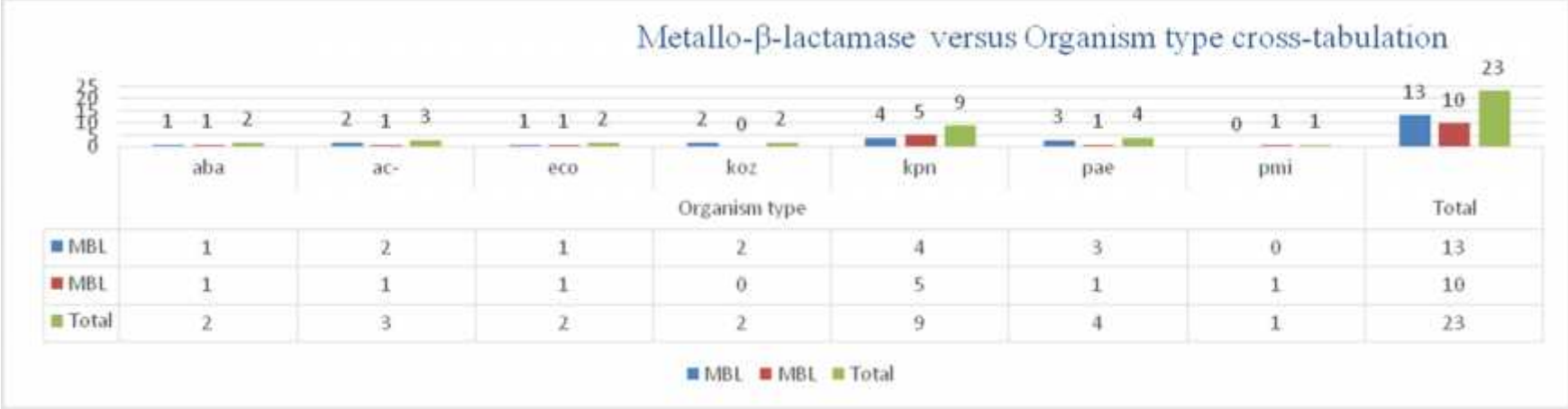


Figure 12: Metallo-β-lactamase versus Organism type cross-tabulation

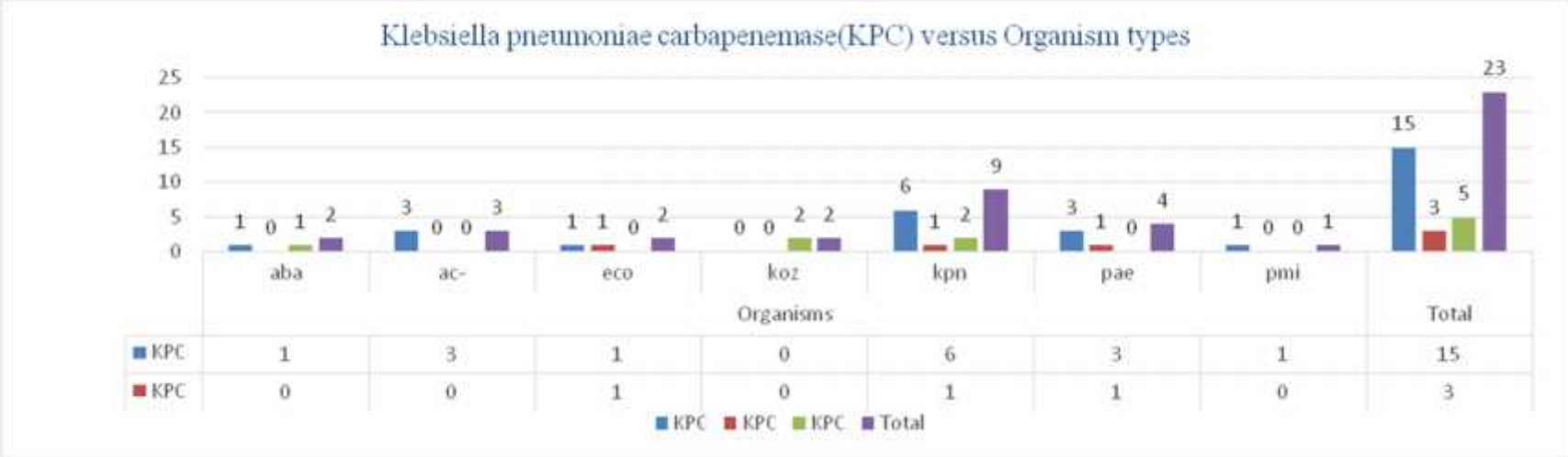


Figure 13: Klebsiella pneumoniae carbapenemase (KPC) versus Organism types cross-tabulation

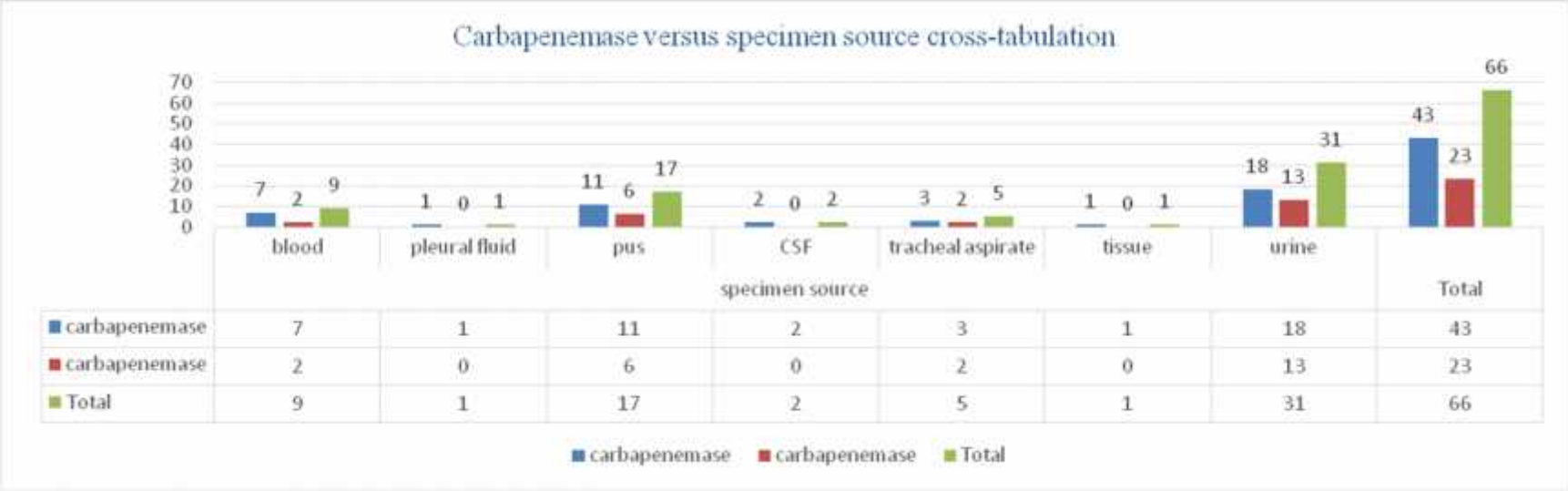


Figure 14: Carbapenemase versus specimen source cross-tabulation

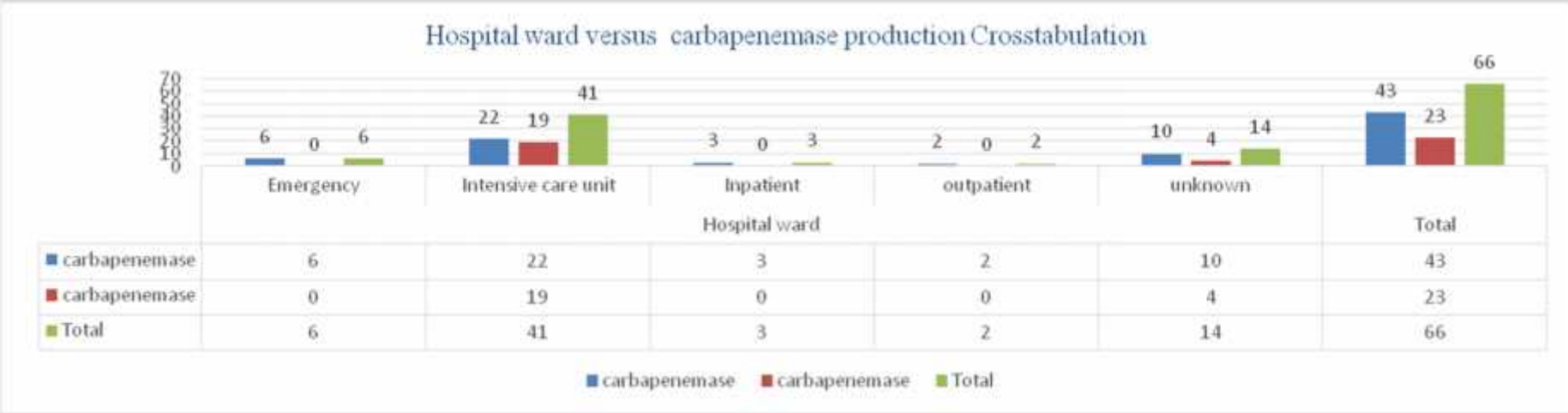


Figure 15:Carbapenemase versus hospital ward cross-tabulation

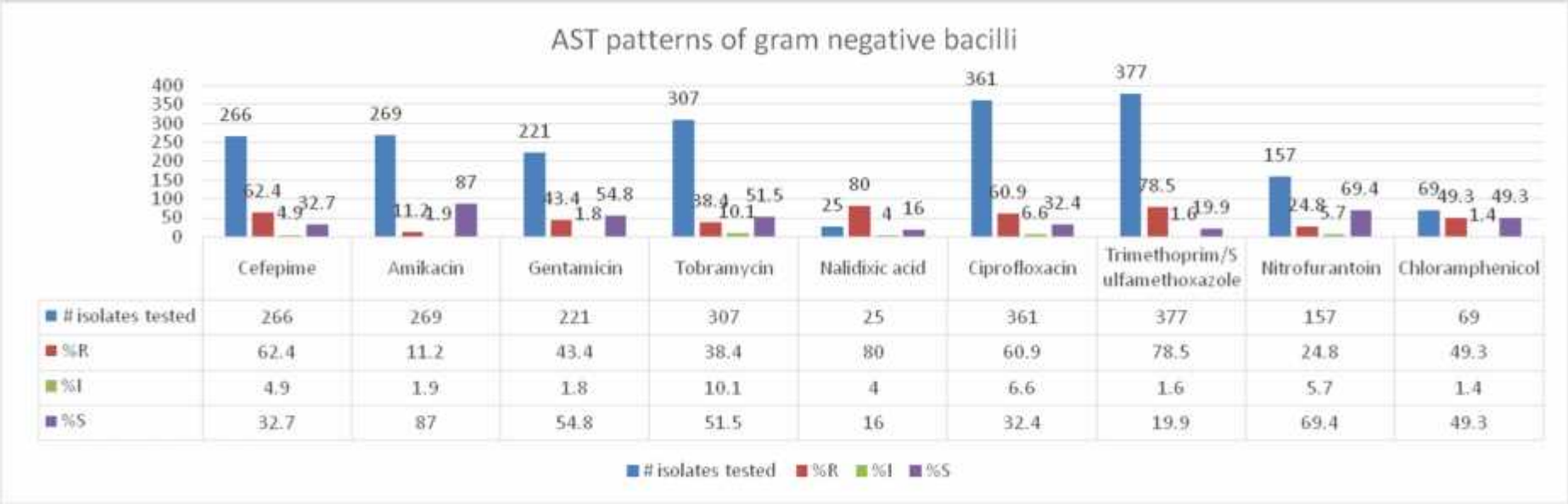


Figure 16: AST patterns of gram-negative bacilli

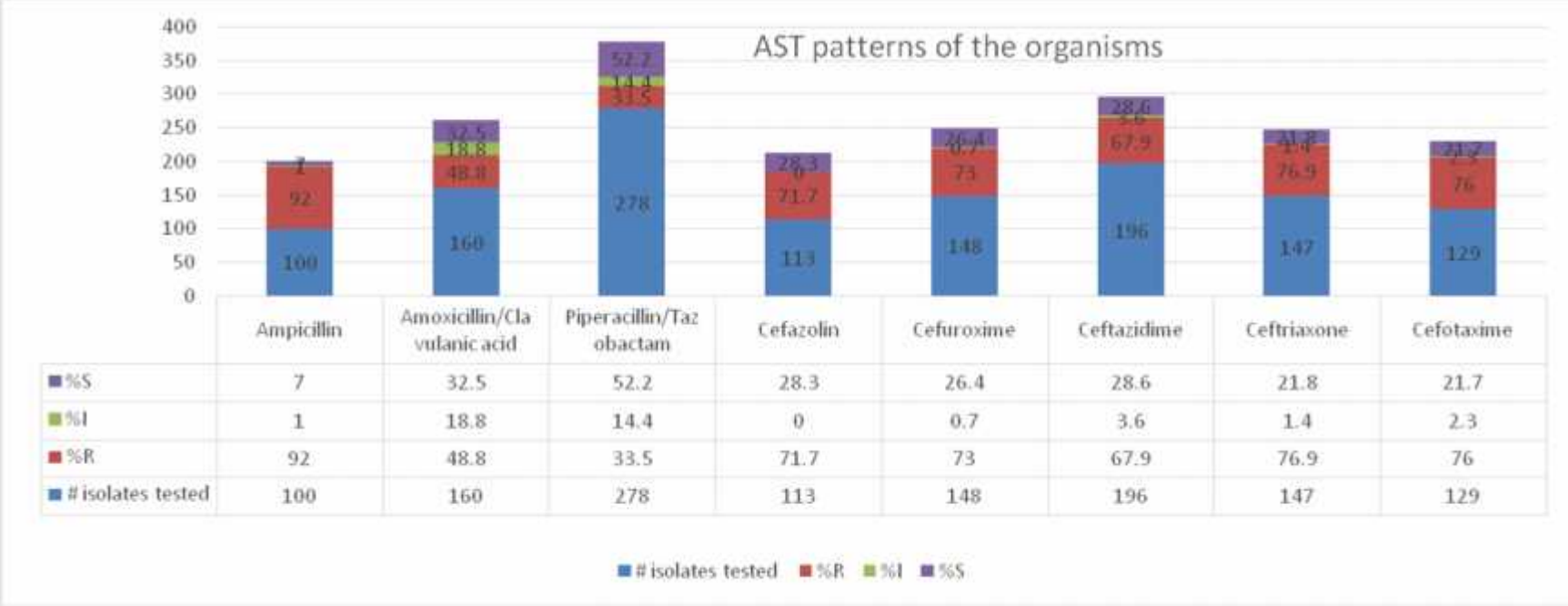


Figure 17: Antimicrobial susceptibility patterns of gram-negative bacilli.

6. Discussion

Infections caused by MDR bacteria, particularly ESBL and carbapenemase-producing gram-negative bacteria are becoming increasingly prevalent and create a potential threat to public health that is associated with high morbidity and mortality rate. Carbapenem antibiotics have an i mportant antibiotic role in that they retain activity against the cephalosporinases and ESBL foun d in gram negative pathogens(68,69).

The rapid emergence of carbapenem hydrolyzing carbapenemase has threatened the clinical utility of this antibiotic class and brings us a step closer to the challenge of "extreme drug resistance" in gram-negative bacilli(68,69). It cleave almost all β-lactams and are plasmid encoded easily transferable among bacterial species(70).

- **ESBL phenotypic assays comparison discussion**

The high prevalence of ESBL among Enterobacteriaceae poses a serious threat to current β lactam antibiotics therapy, leading to treatment failure. The detection of ESBL is potentially cruc ial to ensure adequate therapy and to guide epidemiological and hospital hygiene measures. Laboratory methods for screening and confirmation of ESBL should be relatively cheap, accurate, simple, and rapid(70).

Previous studies In Ethiopia, have reported the high Prevalence of ESBL producing Enterobacter iaceae from 33.3% to 81%(44–55). To our knowledge, the present study is the first study to compare four commercially available phenotypic assays for the detection of ESBL producing *Enterobacteriaceae*.

The Vitek 2 compact system's ability to detect ESBL production was relatively low, with a sensitivity of 92% to 95% and specificity of 50% to 79% in *E. coli* and *K. pneumoniae*(67–69). The Vitek2 method gives indeterminate results for 25% to 31% of the isolates, depending on the types of cards used(67).In our study, this method shows a sensitivity and specificity of 82.9% and 88.2%, respectively, which is lower than the previous studies and Yielded 3.4% (4/117) non-determinable ESBL results.

Vitek 2 compact ESBL detection has been validated only for *E. coli,* K. *pneumoniae, Klebsiella oxytoca, and p. mirabilis by CLSI and E. coli, Klebsiella* spp.*, P. mirabilis, Salmonella* species, *Shigella* species, *Enterobacter* spp.*, Citrobacter freundii, Morganella morganii, Providencia stuartii, Serratia* spp.*, Hafnia alvei by EUCAST*(56,57).

The high sensitivity of the combination disk method when using two or more extended-spectrum cephalosporins has been previously reported and recommended by both CLSI and EUCAST(57,58,67). Our study agrees with the previous studies and International guidelines such as EUCAST and CLSI that the combination disk when using more than one indicator increases the sensitivity of ESBL detection.

The high sensitivity of the double-disk synergy method when using two or more extended-spectrum cephalosporins has been previously published(66-69).Our study agree with the previous studies that the Double disk synergy method, achieved 97.4% and 97.6% sensitivity and specificity respectively.

The ESBL MIC test strips Cefotaxime/ Cefotaxime with clavulanic acid method yields 11% to 49% indeterminate results, mainly because MICs were above the level of detection(67). In the current study, MIC test strips ceftazidime/Ceftazidime with clavulanic acid and MIC test strips Cefotaxime/ Cefotaxime with clavulanic acid yielded 5.1% (6/117) and 6.8%(8/117) non-determinable ESBL results respectively which is relatively lower than previous studies.

The study conducted in 2007 on *E.coli* and *klebsiella* species isolates, showed the sensitivity/specificity values of 97.3%⁄ 96.9%, 91.8%⁄ 100%, and 91.8%⁄ 100% for double disk synergy tests, Vitek-2 ESBL, and combination disk method respectively(71). Which is almost similar for double disk synergy tests and different for Vitek 2 compact and combination disk method when compared to our study.

The study conducted in 2017 in Ethiopia to compare double disk synergy and combination disk method showed, 91.7%, 91.0 and 100% overall, positive and negative percent agreement respectively(72).

Magnitude of carbapenemase producing gram negative bacilli discussion

Even though data are limited for some regions, the overall prevalence of infections caused by carbapenem-resistant bacteria is similar in most continents with non-fermenters being the trickiest pathogens followed by Carbapenem-resistant *Enterobacteriaceae*.The reported burden of carbapenem resistance among gram-negative bacilli across the continent was significantly higher for non-fermenters (mostly>60%) than for fermenters (mostly >10%)(73).

The present study almost agrees with the global burden of carbapenem-resistant bacteria that Carbapenem resistance was highest among non-fermenters, out of 48 carbapenem resistance organisms, 66.67% (32/48) were non-fermenters.

The high burden of Multi-drug resistant gram-negative bacilli ranging from 30.2%, 51.5%, 52.4%, 68.3%, 73.9%, 75.5%, 80%, 81.5%, 94.5% have been reported in Ethiopia(45,45,48,74-79). The magnitudes of Multi-drug resistant gram-negative bacilli in the present studyis (23.77%) thatis lower when compared to other previous studies conducted in Ethiopia.

ASystematic review of about nine studies reported before 2012 on the deaths attributable to Carbapenem-resistant Enterobacteriaceae infections revealed that carbapenem resistance was independently associated with 26%–44% of deaths in seven studies caused mainly by carbapenem-resistant *Klebsiella pneumoniae(*80).In Ethiopiano studies have been conducted to determine the magnitude of death due to infctions by carbapenem resistant bacteria.

The European Survey of Carbapenemase-Producing Enterobacteriaceae initiative (81). The surve y revealed that the Carbapenem-resistant Acinetobacter rates in Bloodstream infections were abo ut 33.4 % (81).The Ethiopian Antimicrobial Resistance Surveillance Annual Report from July 20 17-August 2018 reported 81 CRE and 15 carbapenem resistant Acinetobacter species from data Tikur Anbessa Specialized Hospital, which is also higher(82).

Carbapenem resistanceAcinetobacter is dramatically increasing globally about 40– 70% of the isolates are responsible for ICU-acquired infections (74). In our study, all four carbapenemase positive Acinetobacter species were isolated from ICU patients that is relatively higher than the global data.

Previous studies in Ethiopia have reported the prevalence of carbapenemase-producing gram-negative bacteria, 2%, 2.73%, 7.4%, and 12.12% from different clinical specimens(46–48,50,53,55). In this study, 429 clinical isolates identified from different specimen sources were analyzed, out of these 15.4% (66/429) isolates were non-susceptible to meropenem(ug) or/and Imipenem(10ug). 11.2% (48/429) and 84.6% (363/429) isolates were meropenem and/or Imipene m resistant and susceptible respectively. The results of this study is 5.4% (23/429) which is relatively almost similar with previous studies in Ethiopia.

7. Limitation of the study

- The genes responsible for isolates producing ESBL and carbapenemases were not detected by molecular biology methods
- The outcomes of patients from which multidrug-resistant, ESBL,and carbapenemase-producing isolates were not assessed.

8. Conclusion

Phenotypic assays are cheaper, easy to perform, and interpret. In our study, combination disk method using Cefotaxime and ceftazidime with or without clavulanic acid and double disk synergy method was found to be superior method than Vitek 2 compact, ESBL MIC methods for detection of production of ESBL. Hence, using combination disk method may detect more ESBL producing strains.

9. Recommendations

The prevalence of ESBL andcarbapenemase producing gram negative bacilli has been increasing in our setting which worries clinicians to use extended spectrum cephalosporins and carbapenem antibiotics.Hence, we need to have strong infectionprevention control and antibiotic stewardship committee at health facility.Strengthening the established Ethiopian national antimicrobial resistance surveillance is vital for early detection of these dangerous organisms.Rational use of antibiotics and joint efforts of Microbiologists, patients, Pharmacists, Clinicians,and regulatory bodies are needed to fight against the globally spreading antimicrobial resistance.

10.**References**

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11.ANNEXES

Annex 1: Culture media preparation standard operating procedures

- **Blood agar plate media preparation**
    1. Add the above components (40 gm), except sheep blood, to distilled/deionized water and bring volume to 950.0 mL.
    2. Mix thoroughly.
    3. Heat with frequent agitation and boil for 1 min to completely dissolve.
    4. Autoclave for 15 min at 15 psi pressure at 121°C.
    5. Cool to 45°- 50°C.
    6. Aseptically add 50.0 mL of sterile, defibrinated sheep blood.
    7. Mix thoroughly and pour into sterile Petri dishes.
- **MacConkey Agar preparation**
    1. Suspend 49.53 grams of dehydrated medium in 1000 ml purified/distilled water.
    2. Heat to boiling to dissolve the medium completely.
    3. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
    4. Cool to 45-50°C.
    5. Mix well before pouring into sterile Petri plates.

Preparation of MHA

1. Suspend 38 gm of the medium in one liter of distilled water.
2. Heat with frequent agitation and boil for one minute to completely dissolve the medium.
3. Autoclave at 121°C for 15 minutes. Cool to room temperature.
4. Pour cooled Mueller Hinton Agar 25ml, 60ml for 100mm and 150mm respectively into sterile petri dishes on a level, horizontal surface to give uniform depth.
5. Allow to cool to room temperature.
6. Check for the final pH 7.3 ± 0.1 at 25ºC.
7. Store the plates at 2-8 ºC.

Quality control of MHA

Positive controls:	**Expected results**
Escherichia coli ATCC® 25922	Good growth; pale straw coloured colonies
Pseudomonas aeruginosa ATCC® 27853	Good growth; straw coloured colonies
Staphylococcus aureus ATCC® 25923	Good growth; cream coloured colonies
Negative control:	
Un-Inoculated medium	No change

Preparation of tryptic soy broth

- ✓ Suspend 30 g of medium in 1000 ml of demineralized water.
- ✓ Warm slightly to dissolve completely.
- ✓ Dispense 2ml of medium in to test tube and sterilize at 121°C for 15 minutes or following established laboratory procedures.

Annex 2: ESBL and Carbapenemase detection standard operating procedures

- **Combination disk method for confirmation of ESBL.**

1. Using a fresh, pure culture prepare a suspension of the test organism equal to 0.5 McFarland Standard.
2. Using a sterile cotton swab, spread the adjusted suspension over the entire area of a Mueller Hinton agar plate.
3. Apply discs containing cephalosporin alone (cefotaxime and ceftazidime) and in combination with clavulanic acid.
4. Incubate at 35±2°C for 18-24 hours.
5. Compare the inhibition zone around the cephalosporin disc combined with clavulanic acid with the zone around the disc with the cephalosporin alone.

6. The test is positive if the inhibition zone diameter is ≥ 5 mm larger with clavulanic acid than without.

- **Double disk synergy method for confirmation of ESBL**

1. Using a fresh, pure culture prepare a suspension of the test organism equal to 0.5 McFarland Standard.
2. Using a sterile cotton swab, spread the adjusted suspension over the entire area of a Mueller Hinton agar plate
3. Apply Discs containing cephalosporin (cefotaxime or ceftazidime) next to a disc with clavulanic acid, amoxicillin + clavulanic acid
4. Positive result is indicated when the inhibition zones around any of the cephalosporin discs are augmented in the direction of the disc containing clavulanic acid.
5. The distance between the discs is critical and 20 mm center-to-centre has been found to be optimal for cephalosporin 30 µg discs; however, it may be reduced (15 mm) or expanded (30 mm) for strains with very high or low resistance level, respectively

- **MIC Test Strip Technical Sheet ESBL**

Inoculum preparation

- ✓ Suspend well-isolated colonies from an overnight agar plate into saline to achieve a 0.5 McFarland standard turbidity.
- ✓ A confluent or almost confluent lawn of growth will be obtained after incubation, if the inoculum is correct.
- ✓ In order to verify that your procedure gives the correct inoculum density in terms of CFU/mL, performing regular colony counts is recommended.

- **Note:**
  - ✓ Too heavy inocula may affect the results, since excess enzyme may overcome the clavulanic acid component in the test and consequently reduce the ratio of CTX/CTL, CAZ/CAL or FEP/FEL thus delivering a false negative result.
  - ✓ Analogously, too light inocula may affect results since too little enzyme may provide a lower value for CTX, CAZ or FEP, thus reducing the CTX/CTL, CAZ/ CAL and FEP/FEL ratio.

- **Inoculation**
  - ✓ Dip a sterile swab in the broth culture or in a diluted form thereof and squeeze it on the wall of the test tube to eliminate excess liquid.
  - ✓ Alternatively, use a rotation plater to efficiently streak the inoculum over the agar surface. Allow excess moisture to be absorbed so that the surface is completely dry before applying MIC Test Strip ESBL strips.
- **Application**
  - ✓ Apply the strip to the agar surface with the scale facing upwards and code of the strip to the outside of the plate, pressing it with sterile forceps on the surface of the agar and ensure that whole length of the antibiotic gradient is in complete contact with the agar surface.
  - ✓ Once applied, do not move the strip.
- **Incubation**

Incubate the agar plates in an inverted position at 35 ± 2°C for 16-20 hours in ambient atmosphere.

- **Interpretation**

✓ **Positive**

CTX ≥ 0.5 and CTX/CTL ratio ≥ 8 or CAZ ≥ 1 and CAZ/CAL ratio ≥ 8 or "Phantom" zone or deformation of the CTX, CAZ or FEP ellipse.

✓ **Negative**

CTX < 0.5 or CTX/CTL ratio < 8 and CAZ < 1 or CAZ/CAL ratio < 8.

✓ **ND (non determinable)**

CTX > 16 and CTL > 1 and CAZ > 32 and CAL > 4 or when one strip is ESBL negative and the other ND.

- **Standard operating procedures for performing Simplified carbapenem Inactivation method.**

    1. Prepare a 0.5 McFarland standard suspension (using the direct colony suspension method) of E. coli ATCC 25922
    2. Dilute 1:10 in saline and inoculate onto the MHA plate, following the routine disk diffusion procedure.
    3. Allow Plates to dry for 3–10 min
    4. Smear Imipenem disk on 1–3 overnight colonies of the test organisms grown on blood agar to allow one side of the disk to evenly coat with the test bacteria
    5. Immediately afterward, place the side of the disk having bacteria on the MHA plate previously inoculated with E. coli ATCC 25922.
    6. Place An imipenem disk on MHA plate as the control
    7. Incubate plates at 35◦C for 16–18 h in ambient air
    8. Bacterial strains that produced carbapenemase can hydrolyze imipenem; hence. the susceptible indicator strain grew unchecked. In contrast, the zone of inhibition around the disk shows a diameter of 6– 20 mm , or the satellite growth of colonies of E. coli ATCC 25922 around the disk with a zone diameter ≤22 mm , indicating that the isolate was capable of producing carbapenemase; a zone of inhibition ≥26 mm was considered to be a negative result; a zone of inhibition of 23–25 mm was considered to be a carbapenemase indeterminate result

- **Standard operating procedures for performing Modified Carbapenem Inactivation Methods**

    1. For each isolate to be tested, emulsify a 1-µL loopful of bacteria for **Enterobacterales** or 10-µL loopful of bacteria for *P. aeruginosa* from an overnight blood agar plate in 2 mL TSB.
    2. Vortex for 10–15 seconds.
    3. Add a 10-µg meropenem disk to each tube using sterile forceps or a single disk dispenser. Ensure the entire disk is immersed in the suspension.
    4. Incubate at 35°C ± 2°C in ambient air for 4 hours ± 15 minutes.
    5. Just before or immediately following completion of the TSB-meropenem disk

suspension incubation, prepare a 0.5 McFarland suspension (using the colony suspension method) of *E. coli* ATCC® 25922 in nutrient broth or saline.

6. Inoculate an MHA plate with *E. coli* ATCC® 25922 as for the routine disk diffusion procedure (see M02[4]) making sure the inoculum suspension preparation and MHA plate inoculation steps are each completed within 15 minutes. Allow the plates to dry for 3–10 minutes before adding the meropenem disks.
7. Remove the meropenem disk from each TSB-meropenem disk suspension using a 10-µL loop by placing the flat side of the loop against the flat edge of the disk and using surface tension to pull the disk out of the liquid. Carefully drag and press the loop along the inside edge of the tube to expel excess liquid from the disk. Continue using the loop to remove the disk from the tube and then place it on the MHA plate previously inoculated with the meropenem-susceptible *E. coli* ATCC® 25922 indicator strain. Disk capacity: 4 disks on a 100 mm MHA plate; 8 disks on a 150 mm MHA plate (see Figure 1).
8. Invert and incubate the MHA plates at 35°C ± 2°C in ambient air for 18–24 hours.
9. Following incubation, measure the zones of inhibition as for the routine disk diffusion method (see M02[4]).

Test interpretation

mCIM

- Carbapenemase positive:
  - ✓ Zone diameter of 6–15 mm or presence of pinpoint colonies within a 16–18 mm zone
  - ✓ If the test isolate produces a carbapenemase, the meropenem in the disk will be hydrolyzed and there will be no inhibition or limited growth inhibition of the meropenem-susceptible *E. coli* ATCC® 25922.

- Carbapenemase negative:
    - ✓ Zone diameter of ≥ 19 mm (clear zone)

If the test isolate does not produce carbapenemase, the meropenem in the disk will not be hydrolyzed and will inhibit growth of the meropenem-susceptible *E. coli* ATCC® 25922.

- Carbapenemase indeterminate:
    - ✓ Zone diameter of 16–18 mm
    - ✓ Zone diameter of ≥ 19 mm and the presence of pinpoint colonies within the zone
    - ✓ The presence or absence of a carbapenemase cannot be confirmed.

eCIM – Interpret only when mCIM test is positive

- Metallo-β-lactamase positive:
    - ✓ A ≥ 5-mm increase in zone diameter for eCIM vs zone diameter for mCIM (eg, mCIM = 6 mm; eCIM = 15 mm; zone diameter difference = 9 mm). For only the eCIM test, ignore pinpoint colonies within any zone of inhibition.
    - ✓ If the test isolate produces a metallo-β-lactamase, the activity of the carbapenemase will be inhibited in the presence of EDTA such that the meropenem in the disk will not be hydrolyzed as efficiently as in the tube without EDTA. The result is inhibition of the meropenem-susceptible *E. coli* and an increase in the zone diameter for the eCIM zone diameter compared with the mCIM zone diameter.
- **Metallo-β-lactamase negative:**

✓ A ≤ 4-mm increase in zone diameter for the eCIM vs zone diameter of mCIM (eg, mCIM = 6 mm; eCIM = 8 mm; zone diameter difference = 2 mm). For only the eCIM test, ignore pinpoint colonies within any zone of inhibition.

✓ If the test isolate produces a serine carbapenemase, the activity of the carbapenemase will not be affected by the presence of EDTA and there will be no or marginal (≤ 4 mm) increase in zone diameter in the presence of EDTA compared with the mCIM zone diameter.

Notes

- For mCIM indeterminate results:
  - ✓ Check test isolate and *E. coli* ATCC® 25922 indicator strain for purity.
  - ✓ Check meropenem disk integrity by confirming acceptable results were obtained when disks were subjected to routine disk diffusion test QC.
  - ✓ Repeat the mCIM and/or eCIM for test isolate and QC strains.

- mCIM only: For some tests, pinpoint colonies of the indicator organism (*E. coli* ATCC® 25922) may be observed within the zone of inhibition. If the colonies are present within a 6- to 18-mm zone of inhibition, the test should be considered carbapenemase positive. If colonies are present within a ≥ 19-mm zone, the test should be considered indeterminant.
  - ✓ eCIM only: Ignore pinpoint colonies within any zone of inhibition. Interpret results strictly based on the difference in zone diameters between the mCIM and eCIM tests.
  - ✓ mCIM negative and eCIM positive results should not occur. If this happens, perform checks as indicated in the first bullet above. If the repeat tests are the same, consider the tests invalid.

  CLSI has currently standardized mCIM for **Enterobacterales** with a 1-µL loopful of bacteria and *P. aeruginosa* 10-µL loopful of bacteria only.

QC recommendations

QC Strain	Organism Characteristic	Expected Result
K. pneumoniae ATCC® BAA-1705™	KPC positive Serine carbapenemase producer	mCIM positive eCIM negative
K. pneumoniae ATCC® BAA-1706™	Carbapenemase negative	mCIM negative
K. pneumoniae ATCC® BAA-2146™*	NDM positive Metallo-β-lactamase producer	mCIM positive eCIM positive

MIC Test Strip Technical Sheet KPC

Inoculum preparation

- ✓ Suspend well-isolated colonies from an overnight agar plate into saline to achieve a 0.5 McFarland standard turbidity (1 Mc-Farland if mucoid).
- ✓ A confluent or almost confluent lawn of growth will be obtained after incubation, if the inoculum is correct.
- ✓ In order to verify that your procedure gives the correct inoculum density in terms of CFU/mL, performing regular colony counts is recommended.

Inoculation

- ✓ Dip a sterile swab in the broth culture or in a diluted form thereof and squeeze it on the wall of the test tube to eliminate excess liquid.
- ✓ Alternatively, use a rotation plater to efficiently streak the inoculum over the agar surface.
- ✓ Allow excess moisture to be absorbed so that the surface is completely dry before applying MIC Test Strip.

Application

- ✓ Apply the strip to the agar surface with the scale facing upwards and code of the strip to the outside of the plate, pressing it with sterile forceps on the surface of the agar and ensure that whole length of the antibiotic gradient is in complete contact with the agar surface.
- ✓ Once applied, do not move the strip.

Incubation

- ✓ Incubate the agar plates in an inverted position at 35 ± 2°C for 16-20 hours in ambient atmosphere.
- ✓ Extend the incubation for up to 48 hours in case of slow growing Gram-negative non-fermenters.

  Interpretation
  - ✓ Ratio of ETP/EBO or MRP/MBO of ≥8 or ≥3 log2 dilutions indicates KPC production.
  - ✓ Phantom zone or deformation of the ellipse is also positive for KPC regardless of the ETP/EBO or MRP/MBO ratio.
  - ✓ Send all KPC positive strains to a reference laboratory for confirmation with genotypic testing.

Annex 3: Standard operating procedures for Antimicrobial susceptibility testing

1. Touch the top of at least 4 to 5 well isolated colonies.
2. Transfer the growth to the tube of TSY broth/Normal saline
3. Emulsify the inoculum on the inside of the tube to avoid clumping of the cells.
4. Adjust the inoculum standard to a 0.5 McFarland
5. Compare turbidity to that in the 0.5 McFararnand and standard using a Nephelometer or paper with black lines or use photometric device. Adjust turbidity of inoculum to match that standard.
6. Visually examine the Mueller Hinton agar plates prior to use or other media according to the organism, ensure plates are: free of visible contamination, poured to a uniform depth of approximately 4mm, not excessively wet, not cracked or dry.
7. Dip a sterile cotton swab into the inoculum. Rotate the swab several times and press firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab.
8. Streak the swab over the entire surface of the Mueller Hinton agar plate or other media according to the organism.
9. Rotate the plate approximately 60^{o} then repeat streaking motion.(3x)
10. Complete inoculation by running the swab around the rim of the agar.
11. Allow any excess moisture on the agar surface to be absorbed prior to applying the antimicrobial disks.
12. Dispense disks to the agar surface with sterile forceps (Disk dispenser) or E-test /MIC strip. As per the CLSI guideline
13. After application, insure that the disk E-test /MIC strip has made complete contact with the agar surface by touching the top of the disk with forceps.
14. Incubate plates inverted at 36±1oC for 16 to 18 hours in ambient air/ under 5% CO_2 depending on the air requirement. Incubation time becomes 24hr in case of S.aureus.
15. After incubation with appropriate time, check that the growth is a confluent lawn.
16. Check that zones are round; not oval, deformed or have jagged edges.

NB: *Sometimes when disks are placed closely together, interaction between antimicrobials may produce distortion of inhibition zones*

17. Measure the diameter of inhibition zones. The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye (Naked eye)
18. If no inhibition is present (confluent growth is present up to the border of the disk), the diameter of the disk should be recorded (6mm)
19. For interpretation of results refer CLSI M100 guideline and home-made charts for each type of organism and antibiotics

Procedural Note

- Avoid using over-heated forceps; do not relocate any disks after contact with the agar.
- Every time you have change new drug please change the desiccant also.
- These assays are highly sensitive to variations in: inoculum density, media formulation, media pH, and incubation conditions.
- In addition, agar diffusion methods are strongly influenced by agar depth, diffusion rate of the antimicrobial agent and growth rate of the specific bacteria. Filter paper disks impregnated with antimicrobial agents are placed on the agar.

12.**Declaration**

I, the undersigned agree to accept responsibility for the scientific ethical and technical conduct of the research project and for the provision of required progress reports as per the terms and conditions of the research publications office.

M.Sc. candidate: **Abera Abdeta (B.Sc.)**

Signature: _____

Date of submission: _____

This thesis has been submitted with our approval as advisors.

Advisor: **Adane Bitew (MSc, PhD)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: **Eyasu Tigabu (MSc, PhD)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: **Surafel Fentaw (BSc, MSc)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia