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Magnitude of Extended-spectrum Beta-lactamase, AmpC Beta-lactamase and Carbapenemase producing gram negative bacilli isolated from clinical specimens at International Clinical Laboratories, Addis Ababa, Ethiopia

By: Saba G/Michael (BSc)

Advisors: Mr. Samuel Kind (MSc, PhD Fellow)

Mr. Kassu Desta (MSc, PhD Fellow)

Collaborator: Mr. Melese Hailu (MSc, PhD Fellow)

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This is to certify that the thesis prepared by Saba G/michael Tekele, entitled:

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_____ Signature _____Date_____

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List of Abbreviations

AMR:	Antimicrobial resistance
AST:	Antibiotic Susceptibility Test
ATCC :	American Type Culture Collection
β-lactamases:	Beta-lactamase
CDC:	Communicable Disease Control
CLSI:	Clinical and Laboratory Standards Institute
CPE:	Carbapenemase producing Enterobacteriacae
CRE:	Carbapenem resistant Enterobacteriaceae
ESBL:	Extended spectrum beta-lactamase
EUCAST:	European Committee of Antimicrobial Susceptibility Testing
3GC :	Third generation cephalosporin-resistant
GNB:	Gram negative bacteria
ICL:	International Clinical Laboratories
ID:	Identification
KPC:	Klebsiella pneumoniae carbapenemase
LPS:	Lipopolysaccharides
MBL:	Metallo-ß-lactamase
MDR:	Multidrug resistance
MHT:	Modified Hodge test
OXA-48:	Oxacillinase-48
PAmpC:	Plasmid mediated AmpC
QC:	Quality control
SOPs:	Standard Operating Procedures
TSB:	Tryptose Soya Broth
WHO:	World Health Organization

Abstract

Background: In Gram negative pathogens, beta-lactamase (β-lactamases) production remains the most important mechanism of antimicrobial resistance. These β-lactamases includes Extended spectrum Beta-lactamases (ESBL), AmpC β-lactamases (AmpC) and Carbapenemases.

Objective: To determine the Magnitude of ESBL, AmpC β-lactamases and Carbapenemase producing gram negative bacilli (GNB) isolated from clinical specimens at International Clinical Laboratories, Addis Ababa, Ethiopia.

Methods: A cross sectional study was carried out on 338 GNB isolates between January to May 2018. The bacteria were isolated from urine, wound, body fluids, sputum, stool, ear and eye discharge using 5% sheep blood agar, MacConkey, Xylose lysine deoxycholate and chocolate agar plates. Bacterial species identification, antimicrobial susceptibility testing and β-lactamases screening were performed using Phoenix system. Potential carbapenemase producers were confirmed by Modefied Hodge test and KPC, MBL, OXA-48 were phenotypically characterized by disc diffusion method. Cefoxitin resistant bacteria were confirmed for AmpC β-lactamases production by AmpC confirmatory disc. ESBLs production was confirmed using a combination disc method. Data was entered and analyzed using SPSS version 20 software.

Results: The predominant GNB was *E. coli* 66.0% (n=224) followed by *K. pneumoniae* 12.1% (n=41). The magnitude of β-lactamases producing GNB was 43.0% (n=144). The overall magnitude of ESBL producing GNB was 38.8% (n=131) while, carbapenemase and AmpC β-lactamases producing GNB were 1.2% (n=4) and 2.4% (n=8) respectively. The majority of β-lactamases producing GNB were isolated from urine specimen 47.5% (n=116). Highest resistance level was seen to ampicilline (75.4%), augumentin (64.0%) and sulfamethoxazole-trimethoprim (55.6%). Multidrug resistance (MDR) level was 73.7%. Of β-lactamases producing GNB, 99.3% were MDR ($P < 0.05$).

Conclusion: The magnitude of β-lactamases producing GNB and MDR were high. Therefore, the emergence of β-lactamases producing GNB requires continuous monitoring & reviewing of antimicrobial policy in hospitals and the country at large. Amikacin, meropenem and imipenem were the most effective antibiotics against β-lactamases producing GNB.

Keywords: Beta-lactamase, Multidrug Resistance, Gram negative bacilli, Extended spectrum β-lactamase, AmpC β-lactamase, Carbapenemase

1. Introduction

1.1. Background

Gram negative bacteria such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter baumannii* are among the most important causes of serious nosocomial and community acquired bacterial infections in humans. Resistance to antimicrobial agents in GNB has become important public health concern [1].

The most commonly used antibiotics against bacterial infections are β-lactam antibiotics. The predominant mechanism for resistance to these groups of drug is the production of β-lactamases. β-lactamases can be developed when bacterial gene mutate continuously in response to overuse or misuse of β-lactam antibiotics. However, emergence of resistance to β-lactam antibiotics has become worldwide problem. This leads to treatment failure, increased severity of illness and increased hospital expenses [2-4].

Beta-lactamases are classified according to their functional groups (Bush-Jacoby-Medeiros, groups 1–4, with many subgroups) or molecular schemes (Ambler, class A–D) [5].

Though there are more than 1000 β-lactamase enzymes, ESBL, AmpC β-lactamases and carbapenemase are the most common types of beta-lactamases produced by GNB which are usually multidrug resistant bacteria [6-8].

Infections caused by ESBL and AmpC producing GNB are treated with carbapenems which are last resort antibiotics. However, resistance to carbapenems occurring mainly due to the production of carbapenemase [9]. Currently, carbapenemases grouped under 2d, 2f or 3 and Ambler classification system, they are categorized as class A, B or D. Additionally, other β-lactamases like AmpCs and ESBL combined with other mechanisms such as porin mutations, efflux pump formation or penicillin binding protein (PBPs) alterations can lead to carbapenem resistance [1, 6].

Beta-lactamase production can be detected in different methods using conventional and automated techniques [10]. Rapid and accurate detection of β-lactamase producers will play an

important role in the infection control and prevention of treatment failure. Therefore, one way to shorten the time consumed by microbiological analyses is automation of the laboratory practice. Automation in microbiology laboratories such as Phoenix 100, Vitek-2, and MicroScan WalkAway 96 Plus are used for on time identification of bacterial species and antimicrobial susceptibility testing [11, 12].

Ability to produce enzymes by GNB causes multiple antibiotic resistances resulting in treatment failure, increased morbidity and mortality. Thus accurate and timely detection of these resistant mechanisms is helpful to determine the prevalence of enzymes and their co-production so as to formulate a policy that will aid empirical treatment. The present study was therefore aimed to determine the magnitude of ESBL, AmpC and Carbapenemase among GNB and its antimicrobial resistance profile to recommend effective use of antibiotics and infection control strategy.

1.2 Statement of the problem

Bacterial infections caused by MDR bacteria are a growing threat worldwide. Infections caused by these bacteria are major cause of morbidity and mortality [13]. MDR gram negative isolates, including ESBL and carbapenemase producing gram negative bacilli are an emerging public health problem, with limited therapeutic options [14].

Since beta-lactam antibiotics were the most prescribed antibiotics to treat infections caused by GNB, β-lactamase production has been the greatest threat to these antibiotics. Resistance due to production of enzyme especially ESBLs is a particular problem for *Enterobacteriaceae* infections, which results multidrug resistant species [1].

Beta-lactamases producers commonly show co-resistance to other antibiotic families such as aminoglycosides and fluorquinolones. According to the European Antibiotic Resistance Net (EARS-Net), resistance to third-generation cephalosporins in *E. coli* has increased in Spain from 0.5% in 2001 to 13.9% in 2013[15].

Recently, GNB that produce both ESBLs and AmpC β-lactamases are increasing worldwide. Since β-lactamases producing GNB not always detected in routine antimicrobial susceptibility tests, these organisms usually show multidrug resistance. Lack of methods to detect such resistance is a serious challenge facing clinical laboratories and this became a main factor in the dissemination of ESBL producers and treatment failures [6, 16].

Carbapenemase producing GNB are common causes of different infections including bacteremia, pneumonia, wound infections and urinary tract infections. Those infections are frequently associated with long hospital stay, treatment failures, high mortality rates. For example, in Brazil mortality caused by carbapenem resistant *P. aeruginosa* infections ranged between 51.2% and 95% [17].

Infectious Diseases Society of America (IDSA) indicated that antimicrobial resistance as one of the greatest threats to human health worldwide. In the US, increased hospital expenses was caused by resistant organisms are estimated between $21 billion and $34 billion annually [18].

According to WHO 2017 report on global priority list of antibiotic resistant bacteria, the experts agreed on grouping the pathogens according to the species and the type of resistance and then stratifying the results in three priority tiers (critical, high and medium). *A. baumannii* (carbapenem resistant), *P. aeroginosa* (carbapenem resistant), *Enterobacteriaceae* (carbapenem resistant, 3GC resistant) are grouped under Priority 1: critical tiers [19].

In Sub-Saharan Africa (SSA), although estimations of the magnitude of the problem of antibacterial resistance is difficult and there is limited capacity for antibiotic resistance detection and surveillance, the existing reports showed a high antibiotic resistance rate to commonly used antibiotics [20].

Some studies have been conducted in Ethiopia on carbapenemase producing *Enterobacteriaceae* at different areas indicated that identifying factors such as, patient hospital stay and rational use of drugs that induce carbapenemase production is essential to control spread of CRE within the community [21,22]. Another studies conducted in Ethiopia showed that the higher prevalence of MDR among gram negative bacterial isolates such as in Addis Ababa, Jimma and Gondar [21, 23, 24].

Current knowledge of the prevalence of MDR gram negative bacteria is important to understand their epidemiology, the disease burden and to strengthen hospital infection control strategy to prevent the spread of these bacteria. Previously, few studies have been conducted in Ethiopia on prevalence of ESBL and carbapenemase producing gram negative bacteria but no study has been conducted to detect the extent of drug resistance of these isolates due to AmpC β-lactamase production. Moreover, one way to shorten the time consumed by microbiological analyses is use of automation; here we have applied phoenix system which is rapid and accurate method for ID and AST. Therefore, it is very important to update the available scarce information with regard to the increment of multidrug resistance and widespread of β-lactamase producing GNB in Ethiopia in general and in the study site in particular.

1.3 Significance of the Study

The result of this study

- ✓ Provides information on the magnitude of MDR due to β-lactamases producing and non-β-lactamases producing gram negative bacilli. This will help for policy makers to amend or develop new infection control programs in local situations.
- ✓ Provide information about antimicrobial resistance pattern of β-lactamases and non- β-lactamases producing GNB with their multidrug resistance level. This can help physicians to select the best alternative antibacterial drug for β-lactamase producing GNB treatment.
- ✓ Provide information on alternative β-lactamases screening and confirmatory tests for microbiologists, because the early detection of these bacteria is important to prevent treatment failure.
- ✓ Provide locally applicable data to guide empirical therapy, helps for the rational use of antibiotics and to plan for infection control and surveillance.

2. Literature reviews

2.1 Gram Negative Bacteria

The reason bacteria are either gram positive or gram negative is due to cell wall structure. Gram stain procedure used to separates bacteria into two broad categories based on structural differences in the cell wall [25].

The gram negative bacteria have additional outer membrane composed by phospholipids and lipopolysaccharides which helps to face the external environment. The highly charged nature of lipopolysaccharides (LPS) confers an overall negative charge to the gram negative cell wall [26]. The outer membrane lipopolysaccharides chemical structure is unique to specific bacterial strain and is responsible for many of the antigenic properties of these strains. The pathogenicity of gram negative bacteria is often associated with the LPS layer of the cell wall [27]. The chemical structure of The chemical structure of

Enterobacteriaceae are family bacteria that are rod-shaped, gram negative, non-spore forming, facultative anaerobes and ferment different carbohydrates to obtain carbon. *Enterobacteriaceae* found primarily in the colon of humans and other animals, many as part of the normal flora. As an important cause of nosocomial and community-acquired bacterial infections, the main stay for treatment of infections caused by the *Enterobacteriaceae* was the beta-lactam group of antibiotics [25, 28].

Non-fermenting gram negative bacteria cannot catabolize glucose. This means no fermenters are unable to ferment. These organisms pose a particular difficulty for the healthcare community as they represent the problem of multidrug resistance to the maximum. Important members are *P. aeroginosa* and *A. baumannii*. These bacteria are niche pathogens that primarily cause opportunistic nosocomial infections. MDR is common and increasing among gram negative non-fermenters [29, 30].

2.2 Characteristics of gram negative bacteria

The cell wall structure of gram negative bacteria is different from gram positive bacteria. For example, gram negative bacteria have a cytoplasmic membrane, a thin peptidoglycan (PG) layer and an outer membrane containing LPS. The space between the cytoplasmic membrane and the outer membrane called the periplasmic space. The periplasmic space contains the loose network of peptidoglycan chains known as as the PG layer [31].

Gram positive bacteria do not have murein due to this there is little chemical resemblance to cell walls. In gram negative cells, the amount of murein has been greatly reduced. Some of it forming a single layered sheet around the cell and the rest forming a gel like substance called the periplasmic gel [32].

2.3 Antimicrobial Resistance

Antimicrobial resistance among GNB, especially *Enterobacteriaceae* cause a major problem in nosocomial infections. These infections are common in immune compromised patients [33].

AMR problem is challenging in low income countries due to poor sanitation, high prevalence of infections, inappropriate uses of antibiotics, over the counter availability of drugs and lack of enough clinical microbiology laboratories which can perform AST [34].

Infections caused by MDR are difficult to treat. Especially MDR strains of *A. baumannii, E. coli, K. pneumoniae, Pseudomonas spp., Salmonella spp., Shigella spp.* and beta-lactamase producing strains of bacteria. These strains produce a β-lactamase which can hydrolyze penicillins, cephalosporins, aztreonam, carbapenemes, and other antibiotics [35].

2.4 Mechanisms of Bacteria Resistant to Drugs

Bacteria which lack the specific target site for the drug and naturally resistant to a particular antimicrobial agent called intrinsic resistant (e.g., mycoplasmas are naturally resistant because of lack of cell walls. Therefore, as the drug is unable to cross the cell wall and cannot reach its binding site. Sometimes bacteria become susceptible to a particular drug on the other day it become resistant to it; this is called acquired resistance. Bacteria acquire resistance to

antibiotics by one of the four mechanisms [36].

Before a drug can enter a bacterial cell, molecules of the drug must first bind to drug binding sites. A chromosomal mutation alters the structure of the drug binding sit due to this the drug cannot bind to the cell, this in turn drug cannot enter the cell, and the organism become resistant to the drug. A chromosomal mutation can also result in an alteration of the structure of the cell membrane, which in turn can change membrane permeability. If the drug is unable to pass through the cell membrane, it cannot reach its target and the organism become resistant to the drug [1,32].

Bacteria can acquire resistant genes by transduction (by the help of bacteriophages) and transformation (uptake naked DNA from the outside of cell). The other mechanism of resistance is by formation of efflux pump. An MDR transporter enables the cell to pump drugs out of the cell before it can damage the cell [37, 38].

Another mechanism in which bacteria become resistant to a drug is by producing an enzyme that destroys or inactivates the drug. Bacteria first should acquire enzymes encoded genes to produce enzyme [39]. The principal way in which bacteria acquire new genes helps to produce an enzyme is by conjugation. During conjugation plasmid containing gene is transferred from one bacteria to another bacteria [40].

2.5 Beta-Lactamases

The most prevalent mechanism of resistance to β-lactams among gram negative bacilli is the production of β-lactamases [41].

When the β-lactam ring is destroyed, the antibiotic unable to kill or inhibit the growth of bacteria. Therefore, an organism that produces a β-lactamase is resistant to antibiotics containing the β-lactam ring. β-lactam antibiotics are the commonly prescribed antibiotics to treat different bacterial infections and bacteria produce β-lactamases as defense mechanism against β-lactam antibiotics [1].

In gram negative organisms, β-lactamases are located in the periplasmic space. Because of their location, β-lactamases produced by gram negative organisms are relatively low level. However, low level of enzyme provides a very efficient mechanism of resistance [42].

There are two types of β-lactamases: penicillinases which destroy the β-lactam ring in penicillins and bacteria that produce penicillinase is resistant to penicillins. Cephalosporinases destroy the β-lactam ring of cephalosporins and organism that produces cephalosporinase is resistant to such class of antibiotics [43].

2.6 Classification of Beta-Lactamases

There are two classification schemes for β-lactamases either based on either the functional characteristics of the enzymes or their primary structure. The first classification is based on protein sequence, whereby the β-lactamases are classified into four molecular classes, A, B, C, and D. ESBLs belong to A and D classes. AmpC is class C enzyme and carbapenemase categorizes in A, B and D classes [44].

The functional classification includes group 1 (class C) cephalosporinases; group 2 (classes A and D) broad-spectrum, inhibitor resistant and ESBL and serine carbapenemases; and group 3(MBL) which can destroy carbapenems [45] (Table 2.1).

Table 2.1 Bush and Ambler classification of beta-lactamase (46)

Ambler Class	Bush-Jacoby-Medeiros group	Active site	Enzyme type	Host organisms	Substrates
A	2b, 2be, 2br, 2c, 2e, 2f	Serine	Broad-spectrum β –lactamases (TEM, SHV) ESBL (TEM, SHV, CTX-M) Carbapenemases (KPC, GES, SME)	*Enterobacteriaceae* and nonfermenters	Ampicillin, cephalothin Penicillins, 3GC All β-lactams
B	3	Zinc-binding thiol group	Carbapenemases (VIM, IMP)	*Enterobacteriaceae* and nonfermenters	All β-lactams
C	1	Serine	AmpC cephamycinases (AmpC)	*Enterobacter* species *Citrobacter* species	Cephamycins, 3GC
D	2d	Serine	AmpC cephamycinases (CMY, DHA, MOX FOX, ACC) Broad-spectrum β-lactamases (OXA) ESBL (OXA) Carbapenemases (OXA)	Enterobacteriaceae Enterobacteriaceae and nonfermenters	Cephamycins, 3GC Oxacillin, Ampicillin, Cephalothin Penicillins, 3GC All β-lactams

2.7 Some Strategies in the war against Drug Resistance

Good infection prevention and control procedures are the first strategy to fight drug resistance. This includes education of healthcare professionals and education of patients. It is important that clinicians not allow themselves to be pressured by patients [32].

They should not prescribe or dispense antibiotics unless they are truly necessary and they have made all efforts to test and confirm which antibiotic their patients are treating should have. Whenever possible, physicians should collect a specimen and send to clinical microbiology laboratory to perform culture and susceptibility testing. In addition, clinicians should prescribe an inexpensive, narrow spectrum and appropriate amount of antibiotic whenever the laboratory results demonstrate that such a drug effectively kills the pathogen [47].

Patients also have role in combating drug resistance by taking their antibiotics in the exact manner in which they are prescribed, never skip doses, never save antibiotics for the next time they get sick and safely throw away leftover medication [48].

To combat the effect of β-lactamases, drug companies have developed special drugs that combine a β-lactam antibiotic with a β-lactamase inhibitor (e.g., clavulanic acid, sulbactam, or tazobactam). The β-lactam inhibitor irreversibly binds to and inactivates the β-lactamase, thus enabling the companion drug to enter the bacterial cell and disrupt cell wall synthesis. Combination drugs include: clavulanic acid combined with amoxicillin, clavulanic acid combined with ticarcillin, sulbactam combined with ampicillin, tazobactam combined with piperacillin [37, 49].

2.8 Prevalence of ESBL, AmpC and Carbapenemase producing gram negative bacilli in different countries

Beta-lactamase mediated resistance is the primery mechanism in the multidrug resistant bacteria throughout the world. Especially, countries where infection prevention and control (IPC) protocols are lacking have become the main center for developing multidrug resistance. The prevalence of carbapenemase producing GNB among clinical isolates varies greatly worldwide and within geographic areas. Different studies showed β-lactamase producing GNB is a growing threat worldwide [7, 44, 50].

A systematic review in African countries [51] showed that about 83 studies conducted in Africa which showed that the prevalence of carbapenemase producer isolates was from 2.3% to 67.7% in North Africa and from 9% to 60% in sub-Saharan Africa. Oxacillinases especially blaOXA-48 was the prevalent type of carbapenemase in Africa.

Based on study conducted in south India [52] on AmpC β-lactamases among GNB were tested for resistance to cefoxitin and other antibiotics by disc diffusion method. Isolates which were non susceptible to 3GC and cefoxitin were tested for the production of AmpC β-lactamases. (47%) were positive for AmpC β-lactamases production from (57%) strains resistant to 3GC as well as out of the 86 AmpC producers, 77.9% were cefoxitin resistant.

The production of β-lactamase is the main mechanism for resistance. Study in India done by Doddaiah *et al* [7] to determine the prevalence of three common enzymes produced by GNB from clinical specimens indicated that out of 378 GNB one or more β-lactamases were observed in 197 isolates. Of these, 33.86%, 14.24% and 18.25% were ESBL, AmpC and carbapenemase respectively.

Study done by Singh *et al* [53] in India was undertaken for phenotypic detection of carbapenamase using MHT. A total of 200 isolates of gram negative bacteria isolated from different clinical specimens, 46 isolates of gram negative bacteria were Modified Hodge test positive isolates. Out of which, 29 isolates were resistant of carbapenem and the remaining 17 isolates were suspected carbapenemase producers.

A cross sectional study conducted by Ouedraogo *et al* [54] in in Burkina Faso on high prevalence of ESBL producing *Enterobacteriaceae*. ESBL producing E*nterobacteriaceae* was detected using double disk synergy method and ESBL was 58%. *E. coli* (67.5 %) and *K. pneumoniae* (26 %) were the predominant ESBL producing isolates.

A hospital based cross sectional study done in Sri Lanka by Fernando *et al* [55] indicated that out of 61 ESBL producing organisms, *E. coli* was (86.8%) followed by *K. pneumoniae* in (13.1%). Meropenem, imipenem and amikacin were the most effective drugs against ESBL producing organisms.

Based on the study conducted by Shivanna *et al* [56] on detection of co-existence of β-lactamases in GNB showed that a total of 200 gram negative isolates, ESBL were observed in (25%) isolates and (17.5%) isolates were found to be AmpC were producers. Co-existance of ESBL and AmpC was 38(19%). Among these isolates, one bacteria were AmpC and MBL producer.

From December 2013 to April 2014 study was conducted in Sierra Leone by Leski *et al* [57] antimicrobial susceptibility testing result demonstrated that 85.7% of *Enterobacteriaceae* isolates were MDR where as 64.3% produced an ESBL. High rate of resistance was observed to sulphonamides (91.4 %), chloramphenicol (72.9 %), gentamycin (72.9 %), ampicillin with sulbactam (51.4 %) and ciprofloxacin (47.1 %) and the principal MDR isolate was *C. freundii*.

According to the study done by Nepal *et al* in Nepal [58] out of 268 cultures positive, *E. coli* and *K. pneumoniae* isolates were the most common ESBL and MBL producers. More than half percentage of *E. coli* and *K. pneumoniae* were MDR. The lowest rate of resistance was seen toward imipenem followed by piperacillin/tazobactam and amikacin.

From May 2009 to December 2010 study conducted in Morocco [59] on *Enterobacteriacea* that produce carbapenemase showed *E. coli* was the frequent isolate followed by *Klebsiella spp* and *Enterobacter spp*. Carbapenemase production seen in (2.8%) of the isolates. Out of ten strains produced OXA-48, *K. pneumoniae* account higher percentage and MBL was produced by this bacteria.

Based on study conducted by Yusuf *et al* [60] in Kano-Nigeria to determine the prevalence and co-production of the two enzymes among *Enterobacteriaceae*. A total of 550 clinical isolates of *Enterobacteriaceae* were screened for both AmpC and ESBLs. The overall prevalence of ESBL and AmpC β-lactamase producing isolates was 15.8% and 11.3% respectively. Co-production of ESBL and AmpC β-lactamase was detected in 6.04% of the isolates. Highest rate of co-production was seen among *Enterobacter spp* (12.5%).

Another study in Nigeria conducted by Oduyebo *et al* [61] aimed to determine carbapenemase production among clinical isolates of *Enterobacteriaceae* using Neo‑Sensitabs showed that the overall prevalence of carbapenemase producing *Enterobacteriaceae* was 12.4%. From 27

carbapenem non‑susceptible isolates, (12.4%) were carbapenemase producers but (2.8%) showed carbapenem resistance due to ESBL production. Of the 22 isolates that were positive for carbapenemase production, (8.5%) were MBL producers, (3.4%) produced OXA-48 as well as (0.5%) produced both MBL and KPC.

A cross sectional study done by Ibrahim *et al* in Nigeria [62] on phenotypic detection of ESBL and carbapenemase co-producing isolates showed 58.0% of the isolates were ESBL producers with higher percentage in *K. pneumoniae* (62.9%). Co-productions of carbapenemase and ESBL were observed in both *E. coli* and *K. pneumoniae*. Carbapenemase producers were commonly found in urine and wound. The finding of the study therefore indicates that co-production of enzymes in a bacterium is worrisome.

Based on a cross sectional study in Benin City study done by Ogefere *et al* [63] on prevalence of AmpC β-lactamase among GNB recovered from clinical specimens indicated that out of the 256 gram negative bacterial isolates, (15.23%) were positive for AmpC β-lactamase. The most prevalent AmpC producer was *P. aeruginosa.*

A cross sectional study conducted in Uganda by Andrew *et al* [64] showed that from 100 identified *Enterobacteriaceae,* the predominating bacteria was *E. coli* (44%). Majority of isolates was obtained from urine specimens. Of the 100 tested bacterial isolates, (89%) were identified as ESBL producing pathogens especially *Klebsiella* spp. were the predominated ESBLs producers.

In 2013, study conducted in Ghana [65] indicated that from the total of 300 *Enterobacteriaceae* isolates 49.3% were ESBLs producers. In this study urine 66.70% was the common source of ESBLs producers. In addition, ESBLs producing isolates had highest resistance to co-trimoxazole, gentamicin, amikacin and ciprofloxacin but all isolates were sensitive to meropenem.

According to the study conducted in Sudan by Dahab *et al* [66] on detection of carbapenemase producing GNB isolated from patients in Khartoum State. 149 GNB were isolated from 147 different clinical specimens. The most predominant GNB isolates was *E. coli* (54.4%), followed by *Klebsiella Spp* (29.5%). More than half percentage of the isolates were resistant to

carbapenems and these isolates was confirmed by MHT then 56% of the resistant isolates were carbapenemase producers.

In developing countries like Ethiopia, infections caused by antibiotic resistant bacteria become a public health threat. Study conducted by Eshetie *et al* in Gondar [22] showed the overall prevalence of MDR *Enterobacteriaceae* was (87.4%). From the total isolates *K. pneumoniae* and *E. coli* were predominant isolates and (2.73%) of the isolates were carbapenemase producers.

According to the cross sectional study conducted in Gondar by Moges *et al* [67] indicated that more than 68% of the isolates were MRD which were non susceptible to two or more antimicrobials from different classes. Out of 172 bacterial isolates high resistance were observed in *Citrobacter spp*. for ampicillin (100%), sulfametoxazole-trimetoprem (90%), tetracycline (80%), chloramphenicol (80%) and gentamicin (50 %).

A cross sectional study conducted at Adama Hospital by Mulisa *et al* [68] on prevalence of ESBL producing *Enterobacteriaceae* from urine, surgical wound swabs, body fluids and ear discharges. Out of 133 bacterial strains isolated, *Enterobacteriaceae* account for 51.1%. The overall prevalence of intra species ESBL producers was 25%. *E. coli* was the leading ESBL producer.

Based on study conducted in Addis Ababa by Legese *et al* [21] showed that 33 *Enterobacteriaceae* were isolated from 322 blood and urine specimens. In addition, majority of *Enterobacteriaceae* (72.7%) were isolated from urine cultures. In this study the overall prevalence of ESBL and CPE was 78.6% and 12.1%, respectively. Among the *Enterobacteriaceae* tested, *E. coli* was the principal ESBL producer whereas *K. oxytoca* and *M. morganii* were carbapenemase producing organisms.

3. Objectives

3.1General objective

- To determine magnitude of Extended-spectrum β-lactamase, AmpC β-lactamase and carbapenemase producing gram negative bacilli isolated from clinical specimens at International Clinical Laboratories, Addis Ababa, Ethiopia.

3.2 Specific objectives

- To determine the magnitude of ESBLs producing gram negative bacilli in different clinical specimens.
- To determine the magnitude and classes of carbapenemase producing gram negative bacilli.
- To assess the magnitude of AmpC β-lactamase producing gram negative bacilli.
- To assess antimicrobial resistance pattern of gram negative bacilli.

4. Hypothesis

The magnitude of ESBL, AmpC and carbapenemase among gram negative bacilli isolated from clinical specimens was different with previous studies conducted in Ethiopia.

5. Materials and Methods

5.1 Study Area

The study was conducted at International Clinical Laboratories, Addis Ababa, Ethiopia. ICL opened its doors in 2004 with the great aim of providing quality laboratory service all over Ethiopia. Based on this target, ICL is now expanding its quality service throughout Addis Ababa and the regional cities Bahirdar, Mekelle, Hawassa, Adama, Gondar, Jimma, Dessie, Diredawa and Harrar.

The main laboratory located in Kera around old Bulgaria Mazoria, Addis Ababa and there are about 14 Patient Service Centers (PSC's) or branches. ICL gives a diagnostic service for both patients requested from government and private health facilities including referral hospitals. As a part of commitment to quality, ICL participates in international quality assurance program with College of American Pathologists (CAP), Randox International Quality Assessment scheme (RIQAS) and CDC three times within one year. ICL with its high technology oriented laboratory system has different departments; Department of Hematology, Serology, Chemistry, Immunology, Microbiology and Molecular Diagnostics, Histopathology and Referrals section.

Microbiology department is the one which is equipped with different instruments and microbiological techniques applied. Phoenix system was one of the automation used for identification and antimicrobial susceptibility testing. Monthly in average 300 clinical specimens are being analyzed and the estimated proportions of gram negative bacilli were 100.

5.2 Study Design and Period

A cross sectional study was conducted on gram negative bacterial isolates which was isolated from different clinical specimens in ICL from January 1 to May 30, 2018.

5.3 Population

5.3.1 Source population

All gram negative bacteria isolated from different clinical specimens in Addis Ababa, Ethiopia during the study period were considered as source population.

5.3.2 Study population

The study population was gram negative bacilli isolated from clinical specimens in ICL during the study period that fulfills the inclusion criteria.

5.4 Inclusion criteria

All consecutive gram negative bacilli isolated from different clinical specimens in ICL during the study period were included.

5.5 Exclusion Criteria

- Duplicate GNB from the same patient.

5.6 Study variable

5.6.1 Dependent variable

- Magnitude of ESBL, Carbapenemase and AmpC β-lactamase producing gram negative bacilli
- Antibiotic Susceptibility pattern

5.6.2 Independent variable

- Age
- Sex
- Type of Specimens
- Health facilities

5.7 Measurement and Data collection

5.7.1 Sample size calculation

The sample size was determined using single population proportion formula: $n = Z^2_{\alpha/2} P (1- P)/ d^2$. The expected prevalence, p=87.4% (0.874) which was taken from Eshetie *et al.,* 2015 study in Gondar [22]. The study used 95 % confidence interval and hence Zα/2=1.96. *d* is the margin of error 0.05.

$n = Z^2_{\alpha/2} P (1- P)/ d^2$

. $n = (1.96)^2 0.874(1- 0.874)/ (0.05)^2 = 168$

Though the calculated sample size was 168, we collected and analyzed 338 isolates as the number of isolate during the study period was increased and in order to get representative finding.

5.7.2 Sampling method

A convenient sampling method was used to select the gram negative bacilli that were isolated from different clinical specimens.

5.7.3 Data collection procedure

The socio-demographic data of patients was recorded using worksheet from the request form. Using data collection sheet; the age and sex of the patient, type of specimen, health facilities, the GNB isolated, the types of β-lactamases and the antibiotics susceptibility pattern of the isolate were recorded. All information was collected by the principal investigator.

5.7.4 Laboratory analyses

5.7.4.1 Isolation and identification of gram negative bacilli

The isolates were obtained after inoculation and incubation at 37^{o}C for 18-24 hours on 5% sheep blood agar, chocolate agar, XLD agar and MacConkey agar plates (Oxoid Ltd, UK) from various clinical specimens such as urine, pus, body fluids, sputum, stool, ear and eye discharge. The recovered colonies were characterized by colony appearance and gram stain. The significant

growth colonies were examined morphologically for size, consistency, shape and ability to ferment lactose.

Bacterial species identification was done using phoenix system (BD Diagnostic Systems, Oxford, UK). The turbidity 0.5 McFarland standards inoculum density of bacteria suspension was prepared from pure colony grown on primary isolation media and inoculated to the appropriate phoenix panel.

The ID portion of the Phoenix panel utilizes a series of conventional, chromogenic and fluorogenic biochemical tests to determine the identification of the organism. The tests are based on microbial utilization and degradation of specific substrates detected by various indicator systems. Acid production is indicated by a change in phenol red indicator when an isolate is able to utilize a carbohydrate substrate. Chromogenic substrates produce a yellow color upon enzymatic hydrolysis of either P-nitrophenyl or P-nitroanilide compounds. Enzymatic hydrolysis of fluorogenic substrates results in the release of a fluorescent coumarin derivative. Organisms that utilize a specific carbon source reduce the resazurin-based indicator. In addition, there are other tests that detect the ability of an organism to hydrolyze, degrade, reduce or otherwise utilize a substrate [69].

5.7.4.2 Antimicrobial Susceptibility Test

Susceptibility testing for 16 antimicrobials namely ceftazidime, cefotaxime, ciprofloxacin, ceftriaxone, cefepime, amoxicillin/clavulanic acid, amikacin, aztreonam, ertapenem, cefoxitin, gentamicin, imipenem, meropenem, ampicillin, sulfametoxazole-trimethoprim, piperacillin/ tazobactam was performed by using phoenix AST panel (AST-N94), which also has screening test for ESBL , AmpC , carbapenemase producing GNB.

The Phoenix AST method is a broth based microdilution test. It utilizes a redox indicator for the detection of organism growth in the presence of an antimicrobial agent. Continuous measurements of changes to the indicator as well as bacterial turbidity are used in the determination of bacterial growth. Each AST panel configuration contains several antimicrobial agents. Organism identification is used in the interpretation of the MIC values of each

antimicrobial agent. Phoenix panels are inoculated with a standardized inoculum. Once inoculated, panels are placed into the instrument and continuously incubated at 35°C. The instrument tests panels every 20 minutes [69].

5.7.4.3 Screening gram negative bacilli for ESBLs, AmpC & carbapenemase Production

GNB which were non susceptible to cefotaxime and/or ceftazidime were included as suspected ESBL producers. In other word isolates that showed an MIC ≥2µg/ml for ceftazidime and/or for cefotaxime were considered as potential ESBL producers. Potential carbapenemase producer were selected when MIC 2-4µg/ml for imipenem or meropenem and/or ≥2µg/ml for ertapenem and MIC >16 µg/ml for cefoxitin were screened as potential AmpC β-lactamase producer [70].

5.7.4.4 Isolate Handling and Storage

Suspected beta-lactamase producing gram negative bacilli isolate was sub cultured on 5% sheep blood agar (Oxoid Ltd, UK) to get fresh colonies for phenotypic analysis of ESBL, Carbapenemase and AmpC β-lactamase. After the isolate was analyzed it is stored at -80^{o}C using Tryptose Soya Broth (TSB) (Oxoid LTD, Basingstoke, Hampshire, England) containing 20% glycerol for future reference or for further analysis [70].

5.7.4.5 Confirmation of ESBLs with Combination Disc Test

A disc of ceftazidime (30 µg) and cefotaxime (30 µg) alone and ceftazidime + clavulanic acid (30 µg/10 µg) and cefotaxime (30 µg) + clavulanic acid (30 µg/10 µg) were placed at a distance of 25 mm, center to center, on a Muller Hinton agar plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards and incubated overnight (18 – 24 hrs) at 37°C. An increase in the inhibition zone diameter of ≥5 mm for a combination disc versus ceftazidime or cefotaxime disk alone was confirmed as ESBL producing bacilli according to CLSI (2017) guidelines [70].

5.7.4.6 Confirmation of Carbapenemase production with Modified Hodge test (MHT)

Carbapenemase production was detected by the MHT which is recommended by CLSI. Positive MHT is seen when the test isolate produces the enzyme and allows growth of a carbapenem susceptible strain (*E. coli* ATCC 25922) towards a carbapenem disk. The result is a characteristic

cloverleaf-like indentation. Quality control was performed with each run using MHT Positive *K. pneumoniae* ATCC BAA-1705 and MHT Negative *K. pneumoniae* ATCC BAA-1706 [71].

5.7.4.7 Phenotypic Characterization of Carbapenemases- Combined disc test

Phenotypic test for qualitative identification and detection of the carbapenemases; KPC/MBL and OXA-48 in *Enterobacteriaceae* was done by disc diffusion method using cartridges of tablets containing 10 μg meropenem alone and in combination with inhibitors of different β-lactamases such Phenylboronic acid (KPC & AmpC inhibitor), Dipicolinic acid (MBL inhibitor) and Cloxacillin (AmpC inhibitor). Inhibitors are added to differentiate between isolates with and without resistance mechanisms. In addition, KPC/MBL and OXA-48 confirm kit (ROSCO, Taastrupgaardsvej 30, DK-2630 Taastrup, Denmark) also contains one cartridge of 30 μg Temocillin tablets to detect OXA-48 [72].

5.7.4.8 Phenotypic Confirmation of AmpC beta-lactamase production with combined disc test

All the cefoxitin non susceptible isolates were checked for the presence of AmpC β-lactamase using four cartridges of disc diffusion tablets (ROSCO, Taastrupgaardsvej 30, DK-2630 Taastrup, Denmark) one cartridge of tablets with cefotaxime, one with ceftazidime and two cartridges of the cephalosporins combined with cloxacillin (AmpC inhibitor). An increase in the inhibition zone diameter of $\geq$5 mm for a combination disc versus ceftazidime or cefotaxime disk alone was confirmed as AmpC β-lactamase producing GNB [73].

5.8 Data Quality Assurance

To maintain the quality of the work from isolate collection up to final bacteria identification and data management, the standard operating procedure of laboratory analysis was strictly followed.

Pre analytical stage

Expiry date of the media, reagents and antibiotic discs was checked before use. The culture media was inspected visually for cracks and thickness and for the presence of freezing, bubbles, and contaminant. Phoenix system was checked for its functionality.

Analytical stage

The prepared culture media was checked for sterility by incubating the five percent of prepared media for overnight and observe for the presence of any growth. Abilities of the prepared media supporting the growth of organisms were checked by inoculating control strains *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 25923), *H. influnzae* (10479) and Salmonella spp. (ATCC 700623). Quality control testing for phoenix machine using ATCC strain was done for each lot of panels.

For ESBL *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 control strains was used in this study. *K. pneumoniae* BAA1705 control strains as positive control and *K.pneumoniae* BAA1706 as negative control was used for carbapenemase detection. *Enterobacter cloacae* (ATCC BAA 1143) and *E. coli* (ATCC 25922) used as positive and negative QC strains for AmpC β-lactamase producing GNB respectively. Before applying the 20% glycerol TSB for storage, it was QC tested for growth of *E. coli* ATCC 25922 standard strains.

Post analytical stage

Data was collected using worksheet**,** entered and analyzed using SPSS version 20 software. Finally, it was rechecked by another person.

5.9 Data Analysis and Interpretation

Data was analyzed using SPSS version 20 software. The descriptive summaries were presented with tables and graphs. Chi-square and binary logistic regression was used to see the relation between dependent variable and independent variables. The strength of association was presented by odds ratio and 95% confidence interval and P-value < 0.05 was considered as statistically significant.

5.10 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, School of Allied Health Sciences, College of Health Sciences; Addis Ababa University. Official permission from the study site was obtained. All results were kept confidential; the patients were not able to be

identified by their name or other personal identifier; rather appropriate coding system was used. The principal investigator communicated to the attending physicians when β-lactamase producing GNB were isolated.

5.11 Dissemination of Results

The result of this study was submitted to the department of Medical Laboratory Sciences, School of Allied Health Sciences, College of Health Sciences; Addis Ababa University. The result will be disseminated to International Clinical Laboratories and will also be presented in different scientific and public health forums. It will also be submitted to peer reviewed journal for publication.

5.12 Operational Definitions

I. **Multidrug Resistance:** when a bacterium is simultaneously non susceptible to three or more drugs belonging to different classes of antibiotics.

II. **Extended spectrum bata-lactamase producing gram negative bacilli**: the bacteria that produce the enzyme that confers resistance to most β-lactam antibiotics, including penicillins, cephalosporins and aztreonam but inhibited by β-lactam/ β-lactamase inhibitor.

III. **Carbapenemase producing gram negative bacilli:** the bacteria that produce enzymes that are able to hydrolyze carbapenems or non-susceptible to imipenem, meropenem (MIC 2-4 µg/ml) and ertapenem (MIC ≥2µg/ml) and showed positive results when confirmed by phenotypic test.

IV. **AmpC producing gram negative bacilli:** the bacteria that produce the enzyme that are non-susceptible to cephalosporins and cefoxitin (MIC >16 µg/ml), and also not inhibited by β-lactam/ β-lactamase inhibitors.

6. Results

6.1 Socio-demographic characteristics

A total of 338 GNB isolates were identified from clinical specimens of different sources of infections during the study period. These isolates were recovered from clinical specimens collected from government (151) and private (187) hospitals. The majority of the GNB were isolated from urine 72.2% (244/338) followed by pus 18.6% (63/338) (**Table 6.1**).

Among the total isolates, 58.3% (n= 197/338) were from females and 41.7% (n=141/338) were collected from males with females to males ratio 1.39:1. From all GNB isolated from females, *E. coli* (68.5%) and *K. pneumoniae* (14.2%) were the most frequently isolated bacteria. Similarly among males *E. coli* (63.1%) and *K. pneumoniae* (9.2%) were predominant isolates. The majority of isolates 86(25.4%) were obtained from patients above 61 years of age and the mean age was 43.9 years and standard deviation 21.8.

Table 6.1: Distribution of GNB isolate against demographic characteristics and specimen types at international clinical laboratories, January to May 2018.

Gram negative bacilli n (%)												
Variables (Number)		***E.coli***	***K.pneumoniae***	***K.oxytoca***	***K.ozonea***	***Pseudomonas Spps.***	***Enterobacter Spps.***	***Citrobacter Spps.***	***Acinetobacter Spps.***	***P.mirabilis***	***Shigella Spps.***	**Other Isolate**
Sex	Male(141)	89(63.1)	13(9.2)	1(0.7)	0(0.00)	5(3.5)	8(5.7)	7(5.0)	5(3.5)	1(0.7)	2(1.4)	10(7.1)
	Female(197)	135(68.5)	28(14.2)	3(1.5)	1(0.5)	12(6.1)	3(1.5)	3(1.5)	4(2.0)	4(2.0)	0(0.0)	5(2.5)
Age group	≤15(35)	18(51.4)	4(11.4)	3(8.6)	0(0.0)	2(5.7)	0(0.0)	1(2.9)	2(5.7)	3(8.6)	0(0.0)	3(8.6)
	16-<32(73)	52(71.2)	6(8.2)	1(1.4)	1(1.4)	2(2.7)	4(5.5)	0(0.0)	3(4.1)	2(2.7)	1(1.4)	1(1.4)
	32-<46(74)	49(66.2)	9(12.2)	0(0.0)	0(0.0)	3(4.1)	4(5.4)	4(5.4)	2(2.7)	0(0.0)	1(1.4)	2(2.7)
	46-<61(70)	43(61.4)	10(14.3)	0(0.0)	0(0.0)	7(10.0)	1(1.4)	2(2.9)	1(1.4)	0(0.0)	0(0.0)	6(8.6)
	≥61(86)	62(72.1)	12(14.0)	0(0.0)	0(0.0)	3(3.5)	2(2.3)	3(3.5)	1(1.2)	0(0.0)	0(0.0)	3(3.5)
Types of Specimen	Urine(244)	189(77.5)	23(9.4)	1(0.4)	0(0.0)	4(1.6)	5(2.0)	8(3.3)	4(1.6)	2(0.8)	0(0.0)	8(3.3)
	Pus (63)	24(38.1)	11(17.5)	2(3.2)	0(0.0)	7(11.1)	6(9.5)	2(3.2)	4(6.3)	1(1.6)	0(0.0)	6(9.5)
	Body fluid(10)	7(70.0)	2(20.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(10.0)
	Discharge(13)	1(7.7)	3(23.1)	1(7.7)	1(100)	5(38.5)	0(0.0)	0(0.0)	1(7.7)	2(15.4)	0(0.0)	0(0.0)
	Sputum(6)	3(50.0)	2(33.3)	0(0.0)	0(0.0)	1(16.7)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	Stool(2)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(100)	0(0.0)
	Total (N=338)	**224(66.3)**	**41(12.1)**	**4(1.2)**	**1(0.3)**	**17(5.0)**	**11(3.3)**	**10(3.0)**	**9(2.7)**	**5(1.5)**	**2(0.6)**	**15(4.4)**

Note: *Other isolates are *Salmonella spp*, *Providencia spps, M. morgani* and *Serratia spp.*

6.2 Frequency of gram negative bacilli Isolates

Among all 338 GNB isolates, *E. coli with* 66.3% (224/338) were the most frequent isolates followed by *K. pneumoniae* with 12.1% (41/338). Out of the 224 *E. coli*, 84.4% (189/224) were isolated from urine and 10.7% (24/224) were from pus specimens. From non-fermenters *pseudomonas spp.* were obtained mostly from pus with 41.2% (7/17), followed by discharge and urine with 29.4% (5/17) and 23.5% (4/17) respectively **(Figure 6.1).**

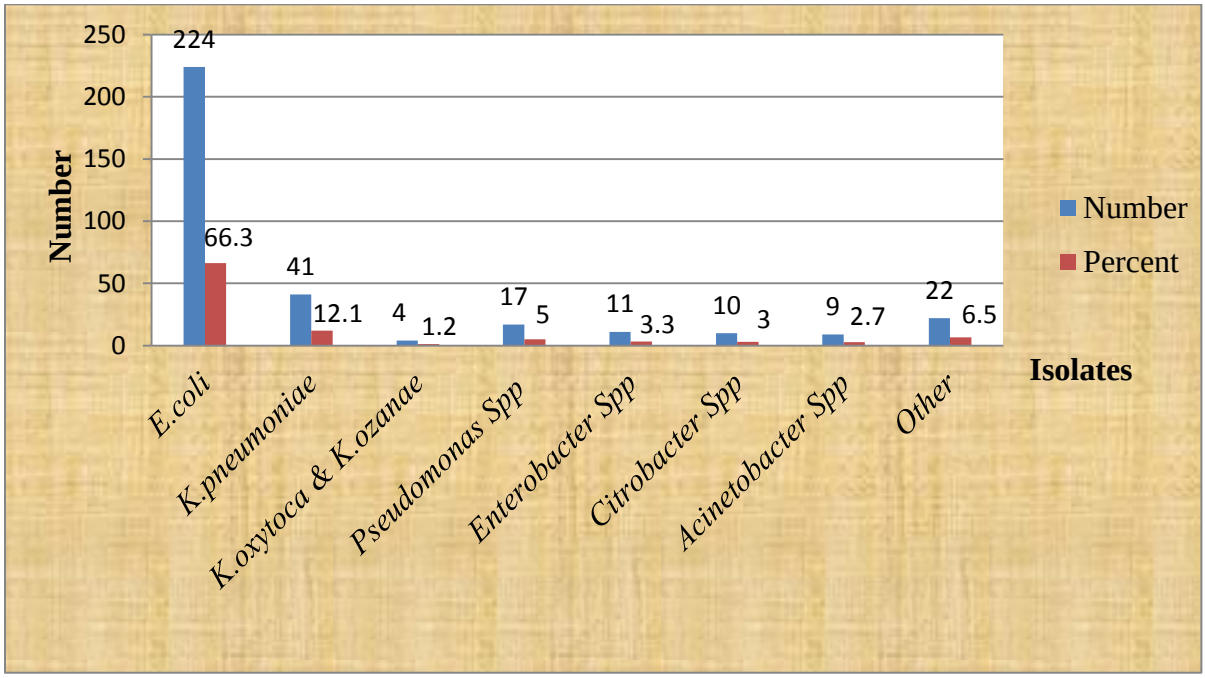


Figure 6.1: Frequency of GNB isolated from different clinical specimens at international clinical laboratories, January to May 2018.

6.3 Antibiotics Resistance pattern of gram negative bacilli

For all isolates the drug susceptibility testing was done by broth microdilution method. Highest resistance level was recorded to ampicillin (75.4%) followed by amoxicillin with clavulanic acid (64.0%), sulfamethoxazole-trimethoprim (55.6%), cefuroxime (48.2%) and cefotaxime (47.0%). *E. coli* showed highest resistance to ampicillin (77.2%) followed by amoxicillin with clavulanic acid (67.9%). In *K. pneumoniae* the highest level of resistance was observed against ampicillin (100%), amoxicillin with clavulanic acid (73.2%), sulfamethoxazole-trimethoprim (70.7%), cefuroxime (65.9%) and azthreonam (63.4%); while no resistance observed to amikacin (0.0%) and low resistance level to meropenem and ertapenem (9.8%), imipenem (12.2%) **(Table 6.2).**

Among the members of GNB isolated *Citrobacter spp and Enterobacter spp* showed 100% resistance to ampicillin. Furthermore, *Serratia spp. and Morganella Spp.* was 100% resistant to amoxicillin with clavulanic acid **(Table 6.2).**

Table 6.2: Antimicrobial resistance pattern of GNB isolated from different clinical specimens at international clinical laboratories, January to May 2018.

Isolates (N)	CRO	CAZ	FEP	CTX	CXM	FOX	MER	IMP	ETP	SXT	CIP	GM	AMP	AMC	AN	ATM	TZP
***E.coli*(n=224)**	110 (49.1)	108 (48.2)	110 (49.1)	114 (50.9)	115 (51.3)	20 (8.9)	2 (0.9)	1 (0.4)	5 (2.2)	135 (60.3)	92 (41.1)	43 (19.2)	173 (77.2)	152 (67.9)	2 (0.9)	114 (50.9)	15 (6.7)
***K.pneumoniae* (n=41)**	26 (63.4)	25 (61.0)	25 (61.0)	26 (63.4)	27 (65.9)	7 (17.1)	4 (9.8)	5 (12.2)	4 (9.8)	29 (70.7)	22 (53.6)	12 (28.6)	41 (100)	30 (73.2)	0 (0.0)	26 (63.4)	8 (19.5)
K.oxytoca(n=4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
***K.ozonea*(n=1))**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
***Pseudomonas Spp.*(n=17)**	NA	4 (23.5)	4 (23.5)	NA	NA	NA	1 (5.9)	0 (0.0)	NA	NA	3 (17.6)	2 (11.8)	NA	NA	2 (11.8)	4 (23.5)	3 (17.6)
***Enterobacter Spp.* (n=11)**	8 (72.7)	5 (45.5)	7 (63.6)	8 (72.7)	8 (72.7)	9 (81.8)	0 (0.0)	0 (0.0)	1 (9.0)	8 (72.7)	5 (45.5)	6 (54.5)	11 (100.0)	11 (100)	0 (0.0)	8 (72.7)	2 (18.2)
***Citrobacter Spp.* (n=10)**	2 (20.0)	2 (20.0)	1 (10.0	5 (50.0)	4 (40.0)	4 (40.0)	0 (0.0)	2 (20.0)	0 (0.0)	6 (60.0)	5 (50.0)	2 (20.0)	10 (100)	9 (90.0)	0 (0.0)	5 (50.0)	1 (10.0)
***Acinetobacter Spp.* (n=9)**	NA	2 (22.2)	3 (33.3)	NA	NA	NA	0 (0.0)	0 (0.0)	NA	NA	2 (22.2)	2 (22.2)	NA	NA	1 (11.1)	NA	2 (22.2)
***P.mirabilis* (n=5)**	1 (20.0)	0 (0.0)	1 (20.0)	1 (20.0)	2 (40.0)	1 (20.0)	1 (20.0)	0 (0.0)	0 (0.0)	2 (40.0)	1 (20.0)	1 (20.0)	2 (40.0)	1 (20.0)	0 (0.0)	1 (20.0)	0 (0.0)
***Shigella Spp* .(n=2)**	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50.0)	0 (0.0)	NA	1 (50.0)	0 (0.0)	NA	0 (0.0)	0 (0.0)
Salmonella Spp. (n=2)	0 (0.0)	NA	NA	1 (50.0)	NA	1 (50.0)	0 (0.0)	1 (50.0)	0 (0.0)	1 (50.0)	1 (50.0)	NA	2 (100)	NA	NA	0 (0.0)	NA
***M. morganii* (n=4)**	3 (75.0)	2 (50.0)	3 (75.0)	3 (75.0)	NA	3 (75.0)	1 (25.0)	1 (25.0)	1 (25.0)	3 (75.0)	2 (50.0)	2 (50.0)	4 (100)	4 (100)	0 (0.0)	1 (25.0)	1 (25.0)
***Providencia spps.* (n=6)**	1 (16.7)	0 (0.0)	0 (0.0)	2 (33.3)	4 (66.7)	1 (16.7)	0 (0.0)	1 (16.7)	2 (33.3)	2 (33.3)	1 (16.7)	1 (16.7)	6 (100)	5 (83.3	1 (16.7)	2 (33.3)	1 (16.7)
Serratia Spp. (n=3)	1 (33.3)	1 (33.3)	0 (0.0)	2 (66.7)	3 (100)	2 (66.7)	0 (0.0)	0 (0.0)	1 (33.3)	1 (33.3)	2 (66.7)	1 (33.3)	3 (100)	3 (100)	1 (33.3)	2 (66.7)	1 (33.3)
Total Resistance(n=338)	**152 (44.9)**	**149 (44.1)**	**154 (45.6)**	**159 (47.0)**	**163 (48.2)**	**49 (14.5)**	**9 (2.7)**	**11 (3.3)**	**14 (4.1)**	**188 (55.6)**	**136 (40.2)**	**72 (21.3)**	**255 (75.4)**	**216 (64.0)**	**7 (2.1)**	**163 (48.2)**	**34 (10.1)**

Note: TZP: pipracillin/tazobactem, GM: gentamicin, AMP: ampicillin, MEM: meropenem, IMP: imipenem, ETP: ertapenem, SXT: trimethoprim–sulfamethoxazole, FOX: Cefoxitin, CRO: ceftriaxone, CXM: cefuroxime, CTX: cefotaxime, CIP: ciprofloxacin, CAZ: ceftazidime, ATM: azthrenam AN: amikacin, AMC: amoxicillin–clavulanic acid, FEP: Cefepime.

6.4 Multi-drug resistance pattern of gram negative bacilli

Among the total isolates (n=338) multi-drug resistance (non-susceptible to at least 3 antibiotics belonging to different antibiotics categories) level were recorded in 249(73.7%) of all bacterial isolates. Relatively higher rate of MDR was seen among *Enterobacter spp.*, *Citrobacter spp.*, *Acinetobacter spp.*, *K. pneumoniae* and *E. coli* accounting average resistance of 90.9%, 90.0%, 88.9%, 82.9% and 69.6% respectively. Surprisingly, the average MDR rate of *Pseudomonas spp.* was found to be 100% and *K. oxytoca* & *K. ozenae* were not MDR isolate.

The most effective antibiotics for MDR were amikacin, meropenem and imipenem with sensitivity of 97.1%, 96.3% and 95.5% respectively **(Table 6.3).**

Table 6.3: Multidrug resistance pattern of GNB isolated from different clinical specimen at international clinical laboratories, January to May 2018.

	Level of antibiotics resistance (n (%))								
Isolates(number)	**R0**	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**≥R7**	**Total MDR isolates(≥3)**
***E.coli*(224)**	28(12.5)	18(8.0)	22(9.8)	33(14.7)	12(5.4)	29(12.9)	33(14.7)	49(21.9)	**156(69.6)**
***K.pneumoniae*(41)**	2(4.9)	3(7.3)	2(4.9)	6(14.6)	2(4.9)	2(4.9)	9(22.0)	15(36.6)	**34(82.9)**
***K.oxytoca* (4)**	2(50.0)	2(50)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	**0(0.0)**
***K.ozanea* (1)**	1(100)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	**0(0.0)**
***Pseudomonas Spp.*(17)**	0(0.0)	0(0.0)	0(0.0)	2(11.8)	1(5.9)	0(0.0)	4(23.5)	10(58.8)	**17(100)**
***Enterobacter Spp.*(11)**	0(0.0)	0(0.0)	1(9.1)	0(0.0)	2(18.2)	0(0.0)	1(9.1)	7(63.7)	**10(90.9)**
***Citrobacter Spp.*(10)**	0(0.0)	0(0.0)	1(10.0)	2(20.0)	2(20.0)	1(10.0)	4(40.0)	0(0.0)	**9(90.0)**
***Acinetobacter Spp.*(9)**	0(0.0)	0(0.0)	1(11.1)	3(33.3)	1(11.1)	1(11.1)	1(11.1)	2(22.2)	**8(88.9)**
***P.mirabilis*(5)**	2(40.0)	0(0.0)	1(20.0)	0(0.0)	1(20.0)	0(0.0)	1(20.0)	0(0.0)	**2(40.0)**
***Shigella Spp.*(2)**	1(50.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(50.0)	0(0.0)	0(0.0)	**1(50.0)**
***Salmonella Spp*(2)**	1(50.0)	1(50.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	**0(0.0)**
***M.morganni*(4)**	2(50.0)	1(25.0)	0(0.0)	1(25.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	**1(25.0)**
***Providentia Spp*(6)**	1(16.7)	3(50.0)	0(0.0)	1(16.7)	0(0.0)	1(16.7)	0(0.0)	0(0.0)	**2(33.3)**
***Serratia Spp*(3)**	1(33.3)	2(66.7)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	**0(0.0)**
Total (n=338)	**38(11.2)**	**25(7.4)**	**31(9.2)**	**45(13.3)**	**23(6.8)**	**36(10.6)**	**54(16.0)**	**88(26.0)**	**249(73.7)**

Note: R0: resistance to no antibiotics, R1-7: resistance to 1, 2, 3, 4, 5, 6, and 7 antibiotics; ≥R3: resistance to 3 or more antibiotics from different classes.

6.5 Magnitude of ESBLs Producing gram negative bacilli

Among 135 ESBL suspected GNB with MIC ≥2µg/ml for ceftazidime and/or for cefotaxime using phoenix system, 38.8% (131/338) were confirmed as ESBLs producing GNB using combination disk test **(Figure 6.2).**

The higher percentage of ESBL producing GNB was recorded in *K. pneumoniae* 56.1% (n=23/41) followed by *E. coli*, *E. cloacae* and *Citrobacter Spp.* with 44.6% (100/224), 36.4% (4/11) and 10.0% (1/10) respectively **(Table 6.4)**. Urine constitute majority of ESBL producing GNB and the proportion of ESBLs was significantly high among isolates from adult patients > 61 years of age (P < 0.05).

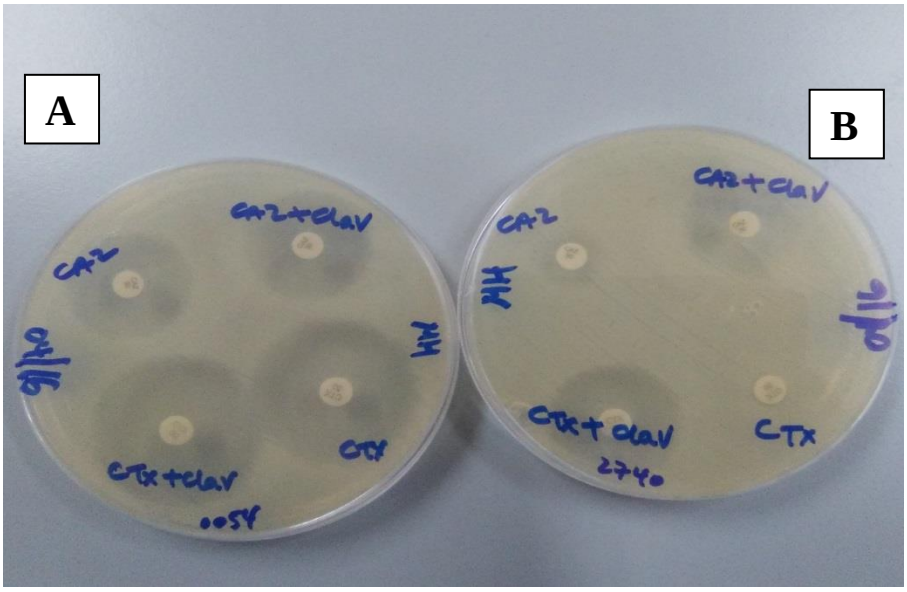


Figure 6.2: ESBL negative (A) and ESBL positive (B) GNB using combination disk test method from different clinical specimens at ICL, January to May 2018.

6.6 Magnitude of Carbapenemase producing gram negative bacilli

Out of the total 338 gram negative isolate enrolled in this study, 17 isolates were potential carbapenemase producers with MIC of 2-4µg/ml for imipenem or meropenem and/or ≥2µg/ml for ertapenem using phoenix system.

Of 17 potential carbapenemase producers, 23.5% (4/17) were confirmed as carbapenemase producer by MHT. The overall magnitude of carbapenemase producing GNB was 1.2% (4/338) which includes *K. pneumoniae* 0.9% (3/338) and *E. coli* 0.3% (1/338) **(Figure 6.3).** All carbapenemase producing bacteria were isolated from urine sample.

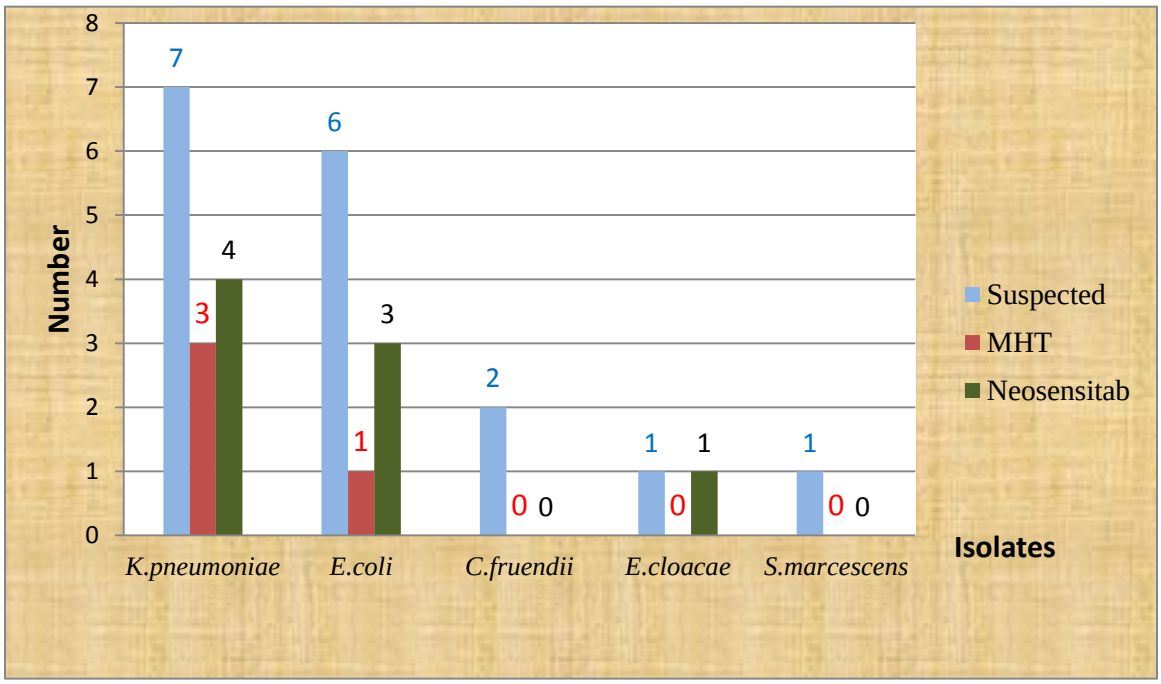


Figure 6.3: Frequency of Carbapenemase producing GNB using two methods from different clinical specimens at international clinical laboratories, January to May 2018.

Similarly those suspected carbapenemase producing GNB (17/338) by phoenix system were phenotypically confirmed and characterized by Neo-Sensitabs™ (Rosco, Denmark) which has sensitivity of 98.8% and specificity of 93.1% according to the user´s guide. Out of 17 carbapenemase suspicious GNB eight was carbapenemase producing GNB by Neo-sensitabs and from these predominant carbapenemase producing GNB, OXA-48 62.5% (5/8), MBL 25.0%(2/8), KPC & OXA-48 12.5%(1/8) was identified type of carbapenemase **(Figure 6.4).** The overall magnitude of carbapenemase producing GNB was 2.4% (8/338) which constitute *K. pneumoniae* 1.2% (4/338), *E. coli* 0.9% (3/338) and *E. cloacae* 0.3% (1/8). The most effective antibiotics against carbapenemase producing GNB were amikacin and ciprofloxacin with 100%

and 75.0% respectively. In carbapenemase producing *K. pneumoniae*, hundred percent resistance was observed to ampicillin and amoxicillin with clavulanic acid.

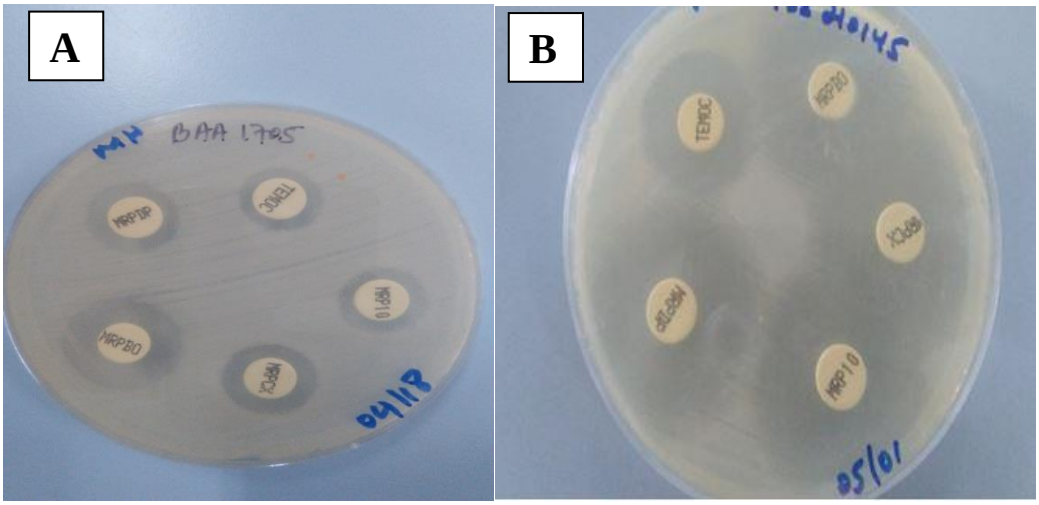


Figure 6.4: KPC positive (A) and Carbapenemase negative (B) GNB using Neo-sensitabs from different clinical specimens at ICL, January to May 2018.

6.7 Magnitude of AmpC producing gram negative bacilli

The overall prevalence of AmpC beta-lactamase producing GNB was 2.4% (8/338) using Neo-Sensitabs™ (Rosco, Denmark) **(Figure 6.5).** *E. coli* 1.2% (4/338), *K. pneumoniae* 0.9% (3/338) and *Citrobacter Spp*. 0.3% (1/338) were AmpC producers among other GNB isolates.

The distribution of AmpC β-lactamase producers varied among different species of GNB. The highest frequency of AmpC β-lactamase production was observed among *K. pneumoniae* 7.3% (3/41) followed by *E. coli and Citrobacter spp.* with 1.8% (4/224) *and* 0.1% (1/10) respectively.

Phoenix system reported 80 cefoxitin resistant or MIC of >16 µg/ml isolates. From these isolates, 10% (8/80) were confirmed as AmpC β-lactamase producing GNB. The most effective antibiotics against AmpC producing GNB was amikacin (100%), meropenem (87.5%), imipenem (85.0%) and gentamycin (80.5%). All AmpC β-lactamase producing GNB were isolated only from urine specimen 3.3% (8/244). This enzyme was more prevalent (40.1%) in age group above sixty one.

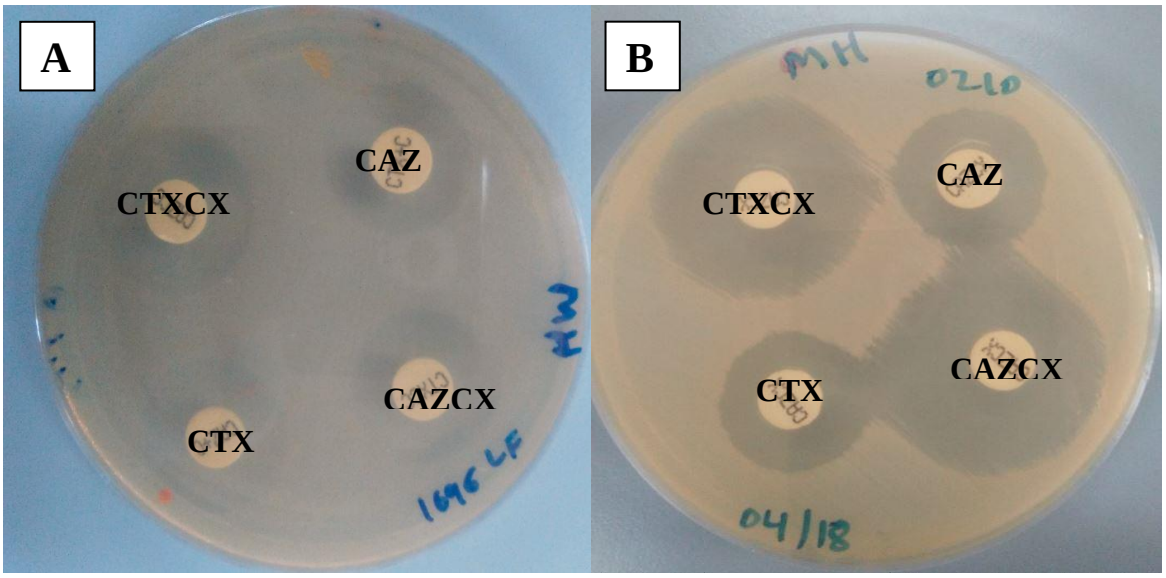


Figure 6.5: AmpC negative (A) and AmpC positive (B) GNB using disk diffusion method from different clinical specimens at ICL, January to May 2018.

In general, β-lactamases mediated MDR in this study was 57.4% (144/249). ESBL, AmpC and Carbapenemase with 38.2%, 2.4% and 1.2% respectively. Neo-Sensitabs™ (Rosco, Denmark) tablet identified 8 (2.4%) carbapenemase producers. Of these, 1.5% (5/338) OXA-48, 0.6% (2/338) MBL and 0.3% (1/338) KPC & OXA-48 were type of carbapenemase identified. The common β-lactamase producing GNB were *K. pneumoniae, E. coli* and *Enterobacter spp.* with 65.9%, 46.9% and 63.6% respectively **(Table 6.4).**

Table 6.4: Distribution of β-lactamases producing GNB & MDR isolate from different clinical specimens at international clinical laboratory, January to May 2018.

Isolates(number)	ESBL	Carbapenemase					AmpC	MDR
		MHT	Neosensitab	OXA-48	MBL	KPC+OXA-48		
***E. coli*(224)**	100(44.6)	1(0.4)	3(1.3)	3(1.3)	0(0.0)	0(0.0)	4(1.8)	155(69.2)
***K. pneumoniae*(41)**	23(56.1)	3(7.3)	4(9.8)	2(4.9)	1(2.4)	1(2.4)	3(7.3)	34(82.9)
***K. oxytoca* (4)**	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
***K. ozanae Spp*(1)**	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
***Pseudomonas Spp*.(17)**	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	17(100)
***Enterobacter Spp*.(11)**	4(36.4)	0(0.0)	1(9.1)	0(0.0)	1(9.1)	0(0.0)	0(0.0)	10(90.9)
***Citrobacter Spp*.(10)**	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(0.1)	9(90.0)
***Acinetobacter Spp*.(9)**	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	8(88.9)
***P.mirabilis*(5)**	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(40.0)
***Shigella Spp*.(2)**	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(50.0)
Other Spp.(15)	3(20.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	13(86.7)
Total (n=338)	**131(38.8)**	**4(1.2)**	**8(2.4)**	**5(1.5)**	**2(0.6)**	**1(0.3)**	**8(2.4)**	**249(73.7)**

6.8 Distribution of beta-lactamase producing gram negative bacilli with their MDR level among different specimens

Among all GNB, 43.0% (144/338) were beta-lactamase producers & 73.7% (n=249/338) were MDR. From the specimen we analyzed, β-lactamase producing GNB 47.5% (116/244) was found predominantly in urine. On the other hand maximum number of MDR was found in wound 81.0% (51/249) (**Table 6.5**). Of β-lactamases producing GNB, 99.3% (n=143/144) were MDR ($P < 0.05$). The rest 0.7% (n=1/144) non-β-lactamases producing GNB were MDR due to other mechanism of resistance. Being β-lactamases producer has statistically significant association with MDR (P=0.001).

Table 6.5: Distribution of β-lactamases producing GNB and MDR level in different clinical specimens at international clinical laboratories, January to May 2018.

Specimens(number)	ESBL	Types of Beta-lactamase n (%)		AmpC	MDR
		Carbapenemase			
		MHT method	Neo S method		
Urine(244)	107(44.0)	4(1.6)	6(2.5)	7(2.9)	181(74.2)
Pus(63)	21(33.3)	0(0.0)	2(3.2)	0(0.0)	51(81.0)
Body fluid(10)	1(10.0)	0(0.0)	0(0.0)	1(0.1)	5(50.0)
Other specimens* (21)	2(9.5)	0(0.0)	0(0.0)	0(0.0)	12(57.1)
Total(n=338)	131(38.8)	4(1.2)	8(2.4)	8(2.4)	249(73.7)

Note: * Other specimens: Sputum (6), stool (4), ear and eye discharge (11)

6.9 Association of independent variables with magnitude of ESBLs, AmpC & Carbapenemase producing gram negative bacilli

Analysis of data using logistic regression model showed that the magnitude of β-lactamases producing GNB had statistically significant association with age group and specimen type. However, there was no statistical significance between sex and health facilities for acquisition of β-lactamase producing GNB (P>0.05). GNB isolate that are isolated from age group >61year are (95%, AOR =1.151 (0.055-0.413), p = 0.000) times more likely to be β-lactamase than other age group. The chances of getting β-lactamase positive among GNB which are isolated from urine specimens are (AOR= 8.015 (1.378, 46.630), P = 0.021) times higher than β-lactamase producing GNB isolated from pus, body fluid and other specimen **(Table 6.6).**

Table 6.6: The association of age, sex, types of specimens and health facilities with magnitude of β-lactamases producing gram negative bacilli at international clinical laboratories, January to May 2018.

Variables		Bivariate analysis		Multivariable analysis	
	BLGNB-n (%)	COR (95% CI)	*P value*	AOR (95% CI)	*P value*
Sex					
Male(141)	73(51.8)	1		1	
Female(197)	71(36.0)	0.295(0.112-0.780)	0.014	0.366(0.134-1.001)	0.150
Age(years)					
≤15(35)	6(17.1)	0.439(0.164-1.179)	0.103	0.477(0.173-1.315)	0.153
16-30(73)	21(28.8)	1		1	
31-45(74)	34(45.9)	0.512(0.186-1.413)	0.196	0.533(0.190-1.495)	0.232
46-60(70)	29(41.4)	0.339(0.117-0.975)	0.045	0.457(0.153-1.366)	0.161
61+(86)	54(62.8)	0.123(0.046-0.327)	0.000	1.151(0.055-0.413)	0.000*
Specimen type					
Urine (244)	115(47.1)	6.375(1.201-33.845)	0.030	8.015(1.378-46.630)	0.021*
Pus (63)	24(38.1)	2.438(0.623-9.530)	0.200	3.024(0.713-12.827)	0.133
Body fluid (10)	2(20.0)	1		1	
Other specimens(21)**	3(14.3)	2.053(0.565-7.463)	0.274	2.363(0.587-9.508)	0.226
Health facilities					
Private (187)	69 (47.9)	1		1	
Government (151)	75 (52.1)	1.188 (0.524-2.689)	0.680	1.65 (0.68-3.99)	0.262

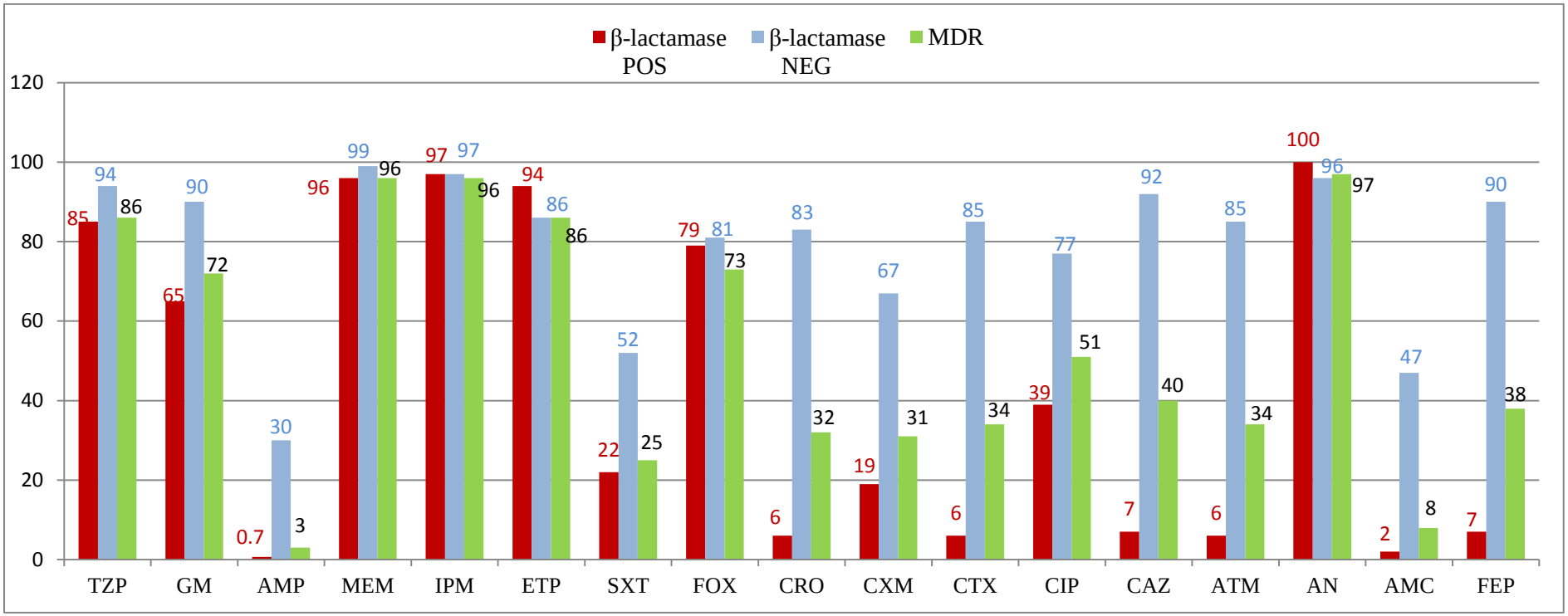
Note: COR: crude odds ratio, AOR: adjusted odds ratio, CI: confidence interval, 1: Reference.

* Statistical significant association between the variables and β-lactamases producing GNB magnitude *(*P<0.05)

**Other specimens are sputum (6), eye and ear discharge (11), stool (4)

6.10 Antibiotics susceptibility of ESBLs, AmpC and Carbapenemase producing gram negative bacilli against different class of antibiotics

Overall non β-lactamase producing GNB were more sensitive to the antibiotics than β-lactamase producers. Amikacin, meropenem, imipenem, ertapenem and pipracillin/tazobactam were the most active drugs for β-lactamase producing GNB with sensitivity of 100%, 95.8, 96.5%, 94.4% and 84.7% respectively. Moreover, 78.5%, 64.6% and 39.0% of β-lactamases producing isolates were sensitive to gentamycin, cefoxitin and ciprofloxacin respectively. On the other side, multidrug resistant GNB were sensitive to amikacin 96.8%, meropenem 96.4%, imipenem 95.6%, pipracilin/tazobactam 86.3% and cefoxitin 72.7% **(Figure 6.6).**



Note: TZP: pipracillin/tazobactem, GM: gentamicin, AMP: ampicillin, MEM: meropenem, IMP: imipenem, ETP: ertapenem, SXT: trimethoprim–sulfamethoxazole, FOX: Cefoxitin, CRO: ceftriaxone, CXM: cefuroxime, CTX: cefotaxime, CIP: ciprofloxacin, CAZ: ceftazidime, ATM: azthrenam AN: amikacin, AMC: amoxicillin–clavulanic acid, FEP: Cefepime.

Figure 6.6: Antibiotics susceptibility pattern of β-lactamases and multi drug resistant GNB to different classes of antibiotics at international clinical laboratories, January to May 2018.

7. Discussions

Beta-lactamases producing GNB have become global threat. Production of β-lactamases with emergence of antibacterial resistance is the most important cause of empirical treatment failures. Most countries have experienced rapid dissemination of β-lactamases producing GNB which cause major therapeutic difficulty. The emergence of β-lactamases producers now a day is challenges to the clinical microbiology laboratories [7].

7.1 Frequency of gram negative bacilli isolates from clinical sample

In the present study, among all GNB isolates, *E. coli* 66.3% (224/338) was predominant isolate followed by *K. pneumoniae* 12.1% (41/338). This finding was comparable with the previous studies carried out in Ethiopia : Bahir Dar, *E. coli* 58.1% and *K. pneumoniae* 23.3% [74], North west Ethiopia : *E. coli* 61.2% & *K. pneumoniae* 15.8% [22]. Similar finding were also reported from other countries; Sudan, *E. coli* 54.4% & *K. pneumoniae* 29.5% [66], Uganda, *E. coli* 53.9 % & *K. pneumoniae* 28.7 % [75], in Southwestern Uganda by Andrew *et al* [64] *E. coli* 44% & *K. pneumonia* 36%, Burkina Faso, *E. coli* 65.6% and *K. pneumoniae* 22.7% [54].

Our finding indicated that *P. aeruginosa* 17(5.0%) were the principal pathogen from non-fermenters. This was in line with previous studies done in Sudan 4.0% [66], Northwest Ethiopia 11.8% [76] but lower than study done in South India 20.4% [52]. This showed that many types of infectious disease were caused by *Enterobacteriaceae* than non-fermenters.

7.2 Antibiotics Resistance pattern of gram negative bacilli

The rate of antimicrobial resistance of identified gram negative isolates was ranging from 0%-75.4%. The present study showed that there were higher resistance to ampicillin (75.4%), followed by amoxicillin with clavulanic acid (64.0%), sulfamethoxazole-trimethoprim (55.6%), aztreonam and cefuroxime (48.8%), cefotaxime (47.0%), cefepime (45.6%), ceftriaxone (44.9%), ceftazidime (44.1%). There were also significant level of resistance to ciprofloxacin (40.2%) and gentamycin (21.3). Comparable result were reported in Ethiopia such as in Gondar: ampicillin (84.6%) and sulfamethoxazole-trimethoprim (79.5%) and gentamicin (35.9%) [22], Debre Markos: ampicillin (70.4%), amoxicillin with clavulanic acid (58.8%), sulfamethoxazole-

trimethoprim (53.1%) [34].However, it was lower than a study conducted in Taznzania: ampicillin (100%), amoxicillin with clavulanic acid (98.7%), sulfamethoxazole-trimethoprim (95.2%), ceftazidime (74.0%) [77], Southeast Iran: sulfamethoxazole-trimethoprim (93.8%) and amoxicillin with clavulanic acid (91.4%) [78] and in Sierra Leone: sulfamethoxazole-trimethoprim (91.4%), gentamycin (72.9%) [57]. The possible reason for this difference might be due to indiscriminate use of antibiotics, patient condition and majority of bacteria in these countries were β-lactamases producing GNB.

In *E. coli* the resistance level to ampicillin (77.2%), amoxicillin with clavulanic acid (67.9%), sulfamethoxazole-trimethoprim (60.3%), cefuroxime (51.3%), aztreonam (51.0%), cefotaxime (50.9%) and ceftriaxone (49.1%) which was in close agreement with study done in South Karnataka: ampicillin (96.2%), cefuroxime (91.6%), amoxicillin with clavulanic acid (77.7%), ceftazidime (75.2%), ceftriaxone (75.1%) and sulfamethoxazole-trimethoprim (67.3%) [79], India: ampicillin (53.3%), amoxicillin with clavulanic acid (46.0%) and gentamycin (39.3%) [80], Dessie: amoxicillin with clavulanic acid (86.0%), gentamicin (79.6%) and ceftriaxone (37.4%) [81], Nepal: sulfamethoxazole-trimethoprim (62.1%), ceftazidime (83.2%), cefotaxime (74.7%), and ciprofloxacin (61.1%) [82].The antimicrobial susceptibility pattern varies from health facility to health facility and also this pattern may vary time to time in the same setting. It might be because of emergence of new resistant strains as a result of inappropriate antibiotics usage [13].

The second most predominant GNB isolates in our study were *K. pneumoniae* which was resistance to most tested antimicrobials such as ampicillin (100%), amoxicillin with clavulanic acid (73.2%), sulfamethoxazole-trimethoprim (70.7%), cefuroxime (65.9%), aztreonam, cefotaxime, ceftriaxone (63.4%), ceftazidime (61.0%). This finding was comparable with study in Gondar: Ampicillin (100%), sulfamethoxazole-trimethoprim (72.2%) [67], in Bahirdar: sulfamethoxazole-trimethoprim (61.0%) [74], in Harer: sulfamethoxazole-trimethoprim (64.9%) [33], Tanzania: ampicillin (100%), amoxicillin with clavulanic acid (90.0%) [49], in Nepal: ampicillin (100%), ceftazidime (86.4%), sulfamethoxazole-trimethoprim (51.3%) [58].

7.3 Multi drug resistance pattern of gram negative bacilli

In the present study, the overall magnitude of MDR among all GNB isolate was 73.7%. There were also similar findings from studies conducted in Gondar (68.0%) [67], Dessie (74.6%) [81], Debre Markos: (72.2%) [34] and Nepal (64.0%) [58]. However, our result was lower than studies done in Sierra Leone (85.7 %) [57], Gondar (87.4%) [22], Bahir Dar (93.1%) [74], Nepal (96.8%) [82].The difference in magnitude of MDR isolates might be due to patient condition, definition for MDR and empirical treatment. In addition, our result was higher when compared to a previous study done in Jimma (59.3%) [23], Nepal (54.2%) [58], another study in Nepal by Lamichhane *et al* [83] reported (33.14%). However, the increased proportion of MDR seen in this study was considered as alarming because only a few treatment options remain for infections. Therefore, implementing strong infection control strategies is required to reduce MDR burden.

The present study showed that *Pseudomonas spp.*(100%), *Enterobacter spp.* (90.0%) and *Citrobacter spp* (90.0%) were found to be the principal MDR isolates which agreed with a study done in Jimma: *Citrobacter spp*. 100% [23], in Nepal: *Enterobacter spp*. 71.4% [83]. Different studies showed different pathogens as a major MDR isolates, in Gondar: *K. pneumonia* (95.6%) and *E. coli* (92.9%) [22], Sierra Leone: *K. pneumoniae* (73.3%) and *E. coli* (61.5%) [57], Nepal: *K. pneumonia* 100% & *E. coli* 95.5% [82] were found to be the predominant MDR isolates. These pathogens are the most commonly found in both hospital and community acquired infections. In addition, these bacteria are intrinsic and acquired resistance to multiple groups of antimicrobial agents, this makes treatment difficult [58].

7.4 Magnitude of ESBLs Producing gram negative bacilli

The overall magnitude of ESBLs producing GNB in the present study was 38.8%, which was in agreement with study reported in Harrer 33.3% [33], Ghana 49.3% [65], Nepal 34.5% [58], Spain 42.8% [84], India 44.0% [56], another study in India by Shashwati *et al* [85] reported 48.3%.

The magnitude of ESBLs producing GNB in our study was lower than studies done in Bahir Dar 57.6% [74], North West Nigeria 58.0% [62], Southwestern Uganda 89% [64] and Southeast Iran

53.8% [78]. This wide variation might be due to differences in study population, sample size, the extent of antibiotic use and improper isolation of patient.

The present study showed the magnitude of ESBLs producing GNB was 38.2%, which was higher than studies reported in Adama 25.0% [68], Nigeria 15.8% [60], Nepal 26.8 % [82] and Italy (6.3%) [86]. This might be anticipated due to good infection control strategy of the countries, study participant, variation in drug management policies and method difference.

E. coli (66.3%) was the most common isolate in present study than *K. pneumoniae* (12.1%) but the predominant ESBLs producing GNB was *K. pneumonia* (56.1%) than *E. coli* (43.8%). This finding was comparable with previous studies done in Bahir Dar: *K. pneumoniae* (69.8%) and *E. coli* (58.2%) [74], North West Nigeria: *K. pneumoniae* (62.9%) and *E. coli* (54.2%) [62], Southwestern Uganda: *K. pneumoniae* (52%) and *E. coli* (44%) [64], Nairobi: *K. pneumoniae* (78.8%) and *E. coli* (60.7%) [87], Uganda: *K. pneumoniae* (72.7 %) and *E. coli* (58.1% [75]**.** These findings demonstrated the predominance of ESBLs production by *K. pneumoniae* than *E. coli*. On the other side, our finding was in contrary to study conducted in Sri Lanka: *E. coli* (86.8%) and *K. pneumoniae* (13.1%) [55], India: *E. coli* (50.14%) and *K. pneumoniae* (48.3%) [85] in which *E. coli* was the predominant ESBLs producer than *K. pneumoniae*.

This study showed ESBL producers among male patients were 48.9% and female 51.1% which was in close agreement with a study done in India 32.3% male and female patients was 67.6% [3] and also the prevalence of ESBLs was significantly higher among isolates from adult patients > 61 years of age (50.2%) than other age groups. Fairly similar finding was observed in Ghana (70.5%) [65] and Burkina Faso (64.7%) [54].

7.5 Magnitude of Carbapenemase producing gram negative bacilli

Although no nationwide study has been conducted so far for detection of carbapenemase producing GNB in Ethiopia, two studies have been done in some parts of the country [21, 22]. The present study showed that out of seventeen carbapenemase suspected GNB, 4(1.2%) were carbapenemase producers using MHT which was in line with study done in Gondar 2.73% [22], Morocco (2.8%) [59] and Jordan 2.8% [88].

The magnitude of carbapenemase producing GNB in current study was lower when compared with the finding in Addis Ababa 12.12% [21]. Uganda 22.4% [89], India 23% [53] and Sudan 56% [66]. The difference in these finding might be due to method difference and the patient condition (in which others only include inpatient and isolates resistant to at least two 3GC). Furthermore, the variation might be due to the difference in local antibiotic prescribing habits and infection control program in different health facilities [90].

In present study 2.4% (8/338) carbapenemase producers were identified using Neo-Sensitabs™ (Rosco, Denmark) tablet. Of these, 1.5% (5/338) OXA-48, 0.6% (2/338) MBL and 0.3% (1/338) KPC & OXA-48 were the type of carbapenemase identified. Therefore, this study showed that OXA-48 enzyme was the most prevalent carbapenemase in Addis Ababa which was supported by study done by Manenzhe *et al*, Oxacillinases especially OXA-48 was the most predominant type of carbapenemase in Africa [51],in Spain 3.2% OXA-48 [84], study conducted in Nigeria, among 22 (12.4%) carbapenemase producers, 15 (8.5%) were MBL producers, 6 (3.4%) produced OXA‑48 [61], in India: MBL was found in 1 (0.5%) isolate [56] and in Nepal 4% were MBL producers [58]. Prevalence and types of carbapenemases can be affected by difference in phenotypic methods, difference in study area and prevalence of carbapenemase genes in different countries [66]. However, surveillance, hand hygiene and appropriate antibiotic usage are part of effective approach in reducing dissemination of these pathogenic organisms [91].

In the current study, co-production of KPC & OXA-48 enzymes were found in one carbapenem resistant isolate. Comparable result was reported in Uganda by Okoche *et al* [89] but this finding was not in agreement with the finding in Nigeria [61] and Thailand [92] which showed co-production of NDM and KPC enzyme in one isolate, since the predominant enzyme in these countries were NDM. Co-production of two carbapenemase enzymes by one bacterium results inactivation of beta-lactamase inhibitors and high level resistance to the carbapenems as well [84].

The present study showed that the most common carbapenemase producing GNB were *K. pneumoniae* (7.3%) followed by *E. coli* (0.4%) which was agreed with study done in Addis Ababa by Legese *et al* [21] *K. pneumoniae* 10.5%, Thailand 4.0% [92] and in Jordan 2.8% [88]. However it was inconsistent compared to the study done in Gondar by Eshetie *et al*, 40.0% [22],

in Sudan by Dahab *et al*, 16.0% [66], in Pakistan by Amjad *et al*, 38.0% [90] reported a principal carbapenemase producing pathogen was *E. coli* than *K. pneumonia.*

In this study, the proportion of carbapenemase producing GNB among female and male was 75% and 25% respectively. Fairly similar result was documented in a study done by Eshetie *et al*, in Gonder: female (66.2%) & male (33.7%) [22], Bangladesh [93] female (58.1%) & male (41.9%). This might be due to increased number of female participants, majority of females were inpatients and females are at high risk of infections especially urinary tract infection.

7.6 Magnitude of AmpC producing gram negative bacilli

AmpC β-lactamases producing GNB have been responsible for several nosocomial outbreaks and high rate of treatment failure [63].

In the present study, from the total specimens, 2.4% (8/338) AmpC producing GNB were isolated. This finding was in line with the study done in Iran 1.5% [94], Greek 2.6% [95], India (8%) [96]. However, it was lower than the finding in Nigeria (15.2%) [63], Spain (14.2%) [84], India (37%) [56], South India (47%) [52]. Differences between these results might be related to geographic regions, detection methods, prevalence of AmpC genes in different countries and study participants.

In present study *K. pneumoniae* 7.3% (3/41), *E. coli* 1.8% (4/224) and *Citrobacter spp*. 0.1% (1/10) was the principal AmpC producing pathogen which was in line with study done by Yimaz *et al*, in Turkey*: K. pneumoniae* 3.6% [97] and Lomero *et al*, in Spain reported *K. pneumoniae* 5.8% were common AmpC producer [84]. However the present study finding was lower than results reported in India: *K. pneumoniae* 22.6% [7], in South India by Parveen *et al*, *K. pneumoniae* 39.1% [52].The possible reason for the difference might be due to sample size, patient condition and geographic area.

The current study also demonstrated co-existence of both ESBL and AmpC enzymes in three isolates 2.2% (3/137) with increased proportion in *E. coli* 3.0% (3/103). This finding was concordant with study conducted in Nigeria (6.04%) [60]. Lower than study done in India [56] in which ESBL and AmpC co-existed in 19% isolates. Simultaneous production of ESBL and AmpC enzymes in a bacterium that causes negative confirmatory test for diagnosis of ESBL

producing isolates or existence of pAmpC enzyme can mask the presence of ESBL [43]. Therefore, simultaneous detection of these enzymes helps to prevent missing of ESBL.

In general, β-lactamases mediated MDR in this study was 57.4% (143/249). ESBL, AmpC and carbapenemase accounted 38.2%, 2.4% and 1.2% respectively. The rest 42.6% MDR (non β-lactamases producing MDR) might be due to activity of efflux pump or lack of outer membrane proteins (OMPs) involved [43]. This was in agreement with study done in India showed the presence of one or more β-lactamases in (52.1%) isolates and (47.8%) were negative. Of these, ESBL (33.8%), AmpC (14.24%) and carbapenemase (18.25%) [7] and Iran (56.8%) of isolates produced at least one type of β-lactamase and ESBL, AmpC and carbapenemase proportion were (52.9%), (7.7%) and (31%) respectively [43] but this was lower than study conducted by Tewari *et al.*, in India [50] showed increased prevalence of ESBL(79.0%), AmpC(34%) and carbapenemase (27.0%). The difference might be due to the factors such as study participant, habit of antibiotic consumption which in turn causes genetic mutations in bacteria producing these enzymes [41], sample size, method difference or poor infection prevention control might also be another reason for difference.

7.7 Distribution of Beta-lactamase producing gram negative bacilli in different specimens

The present study showed that β-lactamases producing GNB were highly found in urine 47.5% (116/244) (AOR= 8.015 (95% CI =1.378, 46.630), p <0.05) followed by pus 38.1% (24/63). This might be due to the larger number of urine samples included in our study. The current study showed maximum ESBL producers 44.0% (107/244) were found in urine. Fairly similar finding was also reported in different countries: Northwest Nigeria 63.5% [62], Uganda 64.9 % [75], Ghana 66.7 % [65], Sierra Leone 64.3 % [57], India 52.3% [85] and in India by Tewari *et al*, 35% [50]. However, study done in Adama, showed that major source of ESBLs producing GNB (53.0%) were isolated from pus [68].The difference might be due to study participant difference or majority of isolates were obtained from pus.

Majority of carbapenemase producing GNB 1.6% (4/244) was found in urine in the present study. Previous studies also showed that the maximum numbers of carbapenem resistant as well as carbapenemase producing isolates were identified from urine sample [66, 21, 87]. However,

this finding was lower than study done in Northwest Nigeria (33.3%) [62], India (39.4%) [53], Spain 34.9% [84].The difference might be due to small sample size and lower magnitude of carbapenemase producing GNB in our study compared to these studies.

The highest magnitude of AmpC producing pathogen in our study was also isolated from urine 2.9% (7/244). This finding was in close agreement with study conducted in Turkey and Nigeria [97, 98] but it was not supported by Ogefere *et al* [63] in Nigeria where isolates from sputum (50.0%) were the predominant producers of AmpC β-lactamase. This indicated that the prevalence of AmpC β-lactamase differs significantly among GNB recovered from different clinical specimens [56].

7.8 Antibiotics susceptibility pattern of ESBLs, AmpC & Carbapenemase producing gram negative bacilli

The present study indicated that the MDR isolates showed maximum resistance to β-Lactam agents, 97.2% & 91.6% resistance was exhibited against ampicillin and amoxicillin with clavulanic acid respectively, comparable to the findings of Eshete *et al*., in Gonder: (100% Vs 97.4%) [22], Kaup *et al*, in South Karnataka (100% Vs 90.2%) [79] and in Sierra Leone 100% resistance to ampicillin [57]. Carbapenems and aminoglycosides were found to be the most effective agents against MDR bacteria [79]. In the present study, MDR isolates showed high level of sensitivity to amikacin (96.8%), meropenem (96.4%), imipenem (95.6%), pipracilin/tazobactam (86.3%) and cefoxitin (72.7%). Comparable result reported in Southeast Iran 99.8% to imipenem [78], Sierra Leone 75.7% to imipenem [57] and in South Karnataka [79] high level of sensitivity to piperacillin/tazobactam (77.2%) reported, study by Kanj *et al*, in USA also indicated that carbapenems were found the most effective agents against MDR [46].

In this study, the highest rate of susceptibility of ESBLs producing isolates were found toward amikacin (100%) followed by imipenem (99.2%), meropenem (97.7%), ertapenem (98.5%), piperacillin/tazobactam (87.0%) and cefoxitine (84.0%). This finding was in harmony with the findings of other studies conducted in Nepal: imipenem (100%), amikacine (86.9%) piperacillin/tazobactam (86.7%) [58], Sri Lanka: meropenem (95%), imipenem (73.7%) and amikacin (60.6%) [55], India: imipenem (100%), piperacillin/tazobactam (89.28%), meropenem (87.5%), and amikacin (83.92%) [85], Ghana: 100% sensitive to meropenem [65], Spain:

imipenem (100%), piperacillin/tazobactam (89.28%), meropenem (87.5%) and amikacin (83.92%) [84] and study by Wadekar *et al*, in shimoga [99] showed 94.5% sensitive to amikacin.

The present study indicated that ESBL producers had high levels of resistance to third generation cephalosporin, penicillin & sulfonamide. Similar finding observed in Iran, Ghana and Uganda [100,65,75]. Highest levels of resistance to ampiciline (99.2%), ceftazidime (98.5%), ceftriaxone (98.5%), amoxicillin with clavulanic acid (98.0%) and sulfamethoxazole-trimethoprim (81.0%) was observed in this study which was agreed with study done in Nepal: sulfamethoxazole-trimethoprim (59%), amoxicillin with clavulanic acid (100%) [58], Nairobi: sulfamethoxazole-trimethoprim (61.1%), ceftazidime (100%), ceftriaxone (100%) [87] and Burkinafaso: sulfamethoxazole-trimethoprim (75 %) [54].

In the present study, the overall antibiotic resistance rates of carbapenemase producing isolates were significantly higher for more than half of tested antibiotics including ampicillin (100%), amoxicillin with clavulanic acid (100%), sulfamethoxazole-trimethoprim (80%), ceftazidime and cefepime (75.0%). This finding was comparable with study in Gondar: ampicillin (100%), sulfamethoxazole-trimethoprim (100%), amoxicillin with clavulanic acid (100%), ceftazidime (80%), gentamycin (80%), cefepime (60%), ceftriaxone 60% [22], Jordan [88] and Bangladish [93] showed including carbapenem drugs, all carbapenemase producing isolates showed highest resistance to amoxicillin with clavulanic (100%), ampicillin (100%), cefepime (100%). These findings showed that carbapenemase producing GNB were the major cause of resistance to various antibiotics classes.

The present study also showed that carbapenemase producing GNB were 100% sensitive to amikacin and 75.0% to ciprofloxacin. This was fairly similar with study conducted in Northwest Nigeria, 57.0% of carbapenemase producing GNB were sensitive to ciprofloxacin [62], Tanzania: ciprofloxacin (66.5%) [77], Nepal: amikacin (91.8%) [58]. Antibiotic treatment options for carbapenem resistant bacteria are limited and are highly costly. However, combination therapy with active drugs such as colistin, tigecycline and flouroquinolones can be alternative antibiotics [46].

Most of the AmpC producing GNB were resistant to the routinely used antibiotics as seen in different studies [43, 52, 60]. In our finding, among cefoxitine resistance isolates, 10% (8/80)

were AmpC producers which was in agreement with the study done in Iran, 5.1% [94], Turkey, 8.7% [97] and India, 37.0% [56]. The possible reasons for cefoxitine resistance might be due to some other resistance mechanism such as, lack of permeability of porins [52].

The present study showed AmpC producers were highly sensitive to imipenem (100%), meropenem (100%) and amikacin (97.0%) and high level of resistance observed in ampicillin (100%), amoxicillin with clavulanic acid (97.0%), ceftriaxone (89.0%), sulfamethoxazole-trimethoprim (75.0%), ceftazidime (68.0%), cefepime (50.0%). This finding was in line with study done in Turkey showed AmpC producers were sensitive to imipenem (100%), meropenem (100%), amikacin (92.0%) and resistance to amoxicillin with clavulanic acid (82.0%), ceftazidime (68.0%), and cefepime (49.0%) [97], Nigeria: AmpC producers were resistant to amoxicillin with clavulanic acid (77.9%), ceftazidime (75.0%) [63], India: resistance to amoxicillin with clavulanic acid (95.9%), sulfamethoxazole-trimethoprim (82.9%), ceftazidime (87.1%) [56].

AmpC producers seems susceptible to cepholosporins in-vitro [101] but when cepholosporins are used in vivo, they result in failure of treatment [102]. Therefore, cepholosporins could not be useful in treating infections caused by AmpC producers. In its place, ofloxacin is the most active agents against AmpC producing GNB.

Generally, this study showed β-lactamase producing GNB were highly resistant to ampicillin (99.3%), amoxicillin with clavulanic acid (98.0%), aztreonam (94.4%), ceftazidime (93.2%), cefepime (93.1%), sulfamethoxazole-trimethoprim (79.0%). This was comparable with study done by shivanna *et al* [56] in India: amoxicillin with clavulanic acid (96.0%), aztreonam (91.3%), ceftazidime (87.1%), sulfamethoxazole- trimethoprim (82.9%), in Iran by Shashwati *et al*, amoxicillin with clavulanic acid (89.3%), sulfamethoxazole-trimethoprim (90.0%) [103], study by Tewari *et al* [50] in India: ceftazidime (97.0%), cefotaxime (76.0%) and in Nepal: amoxicillin with clavulanic acid (100%), ceftazidime (86.4%) [58].

8. Strength and Limitation of the study

8.1 Strength of the study

- This is the first study done on the magnitude of three types of β-lactamase producing GNB from different clinical specimen in Ethiopia.
- The study has tried to characterize carbapenemase phenotypically to show the most prevalent type of carbapenemase in Addis Ababa, Ethiopia.
- Automation for screening and CLSI recommended conventional methods for confirmation of β-lactamase producing GNB were used.
- Neo-Sensitabs™ (Rosco, Denmark) tablets which is highly sensitive and specific, was used for detection of ESBL, Carbapenemase and AmpC β-lactamase producing GNB.
- Antimicrobial Susceptibility testing for 16 antibiotics including β-lactams and β-lactamase inhibitors like piperacillin/tazobactam were tested.
- Isolates are stored at -80ºC for further analysis.

8.2 Limitation of the study

- Blood culture was not included due to Bactec 9050 machine malfunction during the study period.
- Unable to confirm potentially carbapenemase producing non-*Enterobacteriaceae*, because of the method we used do not allow us to confirm for these group of bacteria.

9. Conclusion

This study has demonstrated significant magnitude of β-lactamases producing GNB (ESBL, Carbapenemase and AmpC β-lactamase) and high prevalence of MDR isolates. The majority of β-lactamases producing isolates were found primarily in urine specimen. *K. pneumoniae* and *E. coli* was the most frequent β-lactamases producing GNB. The spread of these organisms reduces the antibiotic alternatives for the treatment of infections caused by these groups' of pathogens to mainly carbapenems; which are often reserved for life threatening infections. Highest level of resistance to multiple classes of antibiotics was observed among β-lactamases producer. The most effective antibiotics for treatment of β-lactamases producing GNB were amikacin, meropenem and imipenem.

10. Recommendations

- Isolation of hospital admitted patients confirmed infection with β-lactamases producing GNB is required to minimize transmission of resistance gene to others.

- Strengthening of antimicrobial resistance surveillance system and effective antibiotic policy like antibiotic restriction, combination therapy and infection control programs combined with good medical practices can help in challenging the risk of antibiotic resistance to prevent the spread of multidrug resistant strains.

- Large scale researches that can assess wide geographical area with large population need to be done.

- Detection of β-lactamases producers need to be introduced as routine tests in microbiology laboratories for rapid detection of resistant isolates and to control their spread, especially for newly admitted patients to the hospitals.

- Molecular analysis recommended in order to confirming the presence of β-lactamases encoding genes.

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ANNEXES

Annex I: In-house Media preparations

1. Tryptose Soy Broth with 20%Glycerol

To prepare TSB media from commercially available dehydrated medium dissolve the recommended amount of powder in distilled water, heat to dissolve, and autoclave at 15 psi at 121ºC for 20 minutes. Its contents are pancreatic digest of casein, Papaic digest of soyabean meal, Sodium chloride, Dipotassium phosphate and Dextrose.

Procedure

1. Suspend 30 gram of powder in 1 liter of distilled water.
2. Adjust pH 7.3+0.2.
3. Warm gently to dissolve.
4. Dispense and autoclave at 121°C for 15 minutes.
5. Then to make 20% glycerol TSB mix 800ml TSB with 200ml glycerol.
   - TSB with 20% glycerol is used for long term storage of the isolates.

2. Preparation of Mueller Hinton Agar

MHA is made from commercially available dehydrated medium. To prepare media from commercially available dehydrated medium dissolve the recommended amount in distilled water, heat to dissolve, and autoclave at 15 psi at 121ºC for 20 minutes. Its contents are Beef extract, Acid digest of casein, Starch.

1. Prepare and sterilize the medium as instructed by the manufacturer.
2. The pH of the medium should be 7.2–7.4.
3. Autoclave at 121°C at 20 minutes. Do not over heat.
4. Pour into 150 mm or 100 mm diameter sterile petri dishes to a depth of 4 mm (about 25 ml per plate). Care must be taken to pour the plates on a level surface so that the depth of the medium is uniform.

Note: If the medium is too thin the inhibition zones will be falsely large and if too thick the zones will be falsely small.

Annex II. Laboratory procedures for bacteria identification, drug susceptibility testing, ESBL, Carbapenemase and AmpC β-lactamase detection and isolate handling.

1. Culture and identification

The isolates was identified by standard microbiological laboratory methods such as colony appearance, gram stain and the pure colony also used for bacterial identification as well as antibiotic susceptibility test using phoenix 100 machine. Isolates suspected of beta-lactamase production was subcultured on blood agar plate for confirmation using phenotypic methods.

I. Identification steps for the isolated colonies

a) Colony Characteristics
b) Gram stain:_____
c) Oxidase test: positive □ Negative □
d) □ Lactose Fermenter □ Non lactose fermenter

II. Identification and AST on BD phoenix100

Intended use

BD phoenix^{100} automated system is used in clinical laboratories for identification of bacterial species and antimicrobial susceptibility testing.

Principle

A maximum of 100 identification and antimicrobial susceptibility tests can be performed in the phoenix instrument at a time using phoenix combination panels. The combination panel includes an ID side with dried substrates for bacterial identification and an AST side with varying concentrations of antimicrobial agents, growth and fluorescent controls at appropriate well locations. The phoenix system utilizes an optimized colorimetric redox indicator for AST, and a variety of colorimetric and fluorometric indicators for ID. The AST broth is cation-adjusted (e.g., Ca++ and Mg++) to optimize susceptibility testing performance. The phoenix panel is comprised of a 51 well ID side and an 85 well AST side. The ID side contains 45 wells with dried biochemical substrates and 2 fluorescent control wells. The AST side potentially contains up to 84 wells with dried antimicrobial agents and 1 growth control well. Phoenix panels are inoculated with a standardized inoculum. Organism suspensions must be prepared only with the

BBL™ CrystalSpec™ nephelometer or the BD Phoenix Spec. Once inoculated, panels are placed into the instrument and continuously incubated at 35°C. The instrument tests panels every 20 minutes: on the hour, at 20 minutes past the hour, and again at 40 minutes past the hour for up to 16 hours if necessary. Phoenix panels are read only by the phoenix instrument. Phoenix panels cannot be read manually.

Procedure

A. General Panel Preparation

1. Confirm the Gram stain reaction of the isolate before proceeding with the inoculum preparation for use in the phoenix instrument. Once the gram stain reaction is confirmed, select the appropriate phoenix panel for inoculation.
2. Examine the pouch, and do not use the panel if the pouch is punctured or opened. Remove the panel from the pouch. Discard the desiccant. Do not use the panel if there is no desiccant or if the desiccant pouch is torn.
3. Place the panel on the Inoculation Station with the inoculation ports on top and the pad on the bottom.
4. Label a Phoenix ID broth tube with the patient's specimen number. Using aseptic technique, pick colonies of the same morphology with the tip of a sterile cotton swab or a wooden applicator stick from one of the recommended media.
5. Suspend the colonies in the Phoenix ID broth (4.5 mL).
6. Cap the tube and vortex for five seconds.
7. Allow approximately ten seconds for air bubbles to surface. You can tap the tube gently to aid in eliminating bubbles.
8. Insert the tube into the BD PhoenixSpec Nephelometer. Make sure the tube is inserted as far as it will go.
9. If the inoculum density is set to 0.5 McFarland for the panel type being run, then a range of 0.50 – 0.60 is acceptable. If the density of organisms is low, you can add colonies from the isolate. Re-vortex the sample and reread to confirm that the correct McFarland has been achieved. If the density of organisms exceeds 0.6 McFarland, follow the steps below to dilute the broth. It is very important to accurately indicate the level of the liquid in the tube since this volume is needed to adequately fill the wells in the panel.

a. Using a marker, mark the broth level in the over inoculated Phoenix ID Broth tube.

b. Using a sterile pipette, aseptically add fresh Phoenix ID Broth to the inoculum. Only Phoenix ID Broth may be used to dilute the inoculum.

c. Vortex the tube and allow to sit for 10 seconds.

d. Place the tube in the nephelometer and re-measure the turbidity of the suspension.

- If the reading is greater than 0.6, repeat Steps b-d.

  If the reading is 0.5 - 0.6, go to Step e.

e. Using a sterile pipette, aseptically remove excess broth to the original level indicated by the mark on the tube created in Step a. Remove excess broth to avoid overfilling the panel. Also, do not remove too much broth, as there may be insufficient broth to adequately fill the panel.

f. Broth may now be used to inoculate the Phoenix AST Broth and/or the Phoenix Panel.

10. If you are performing identification only, proceed to Step 15 and continue the procedure. If you are inoculating a Phoenix Plus Panel, refer to the section below, Phoenix PlusPanels.
11. Label a Phoenix AST broth tube (8.0 mL) with the patient's specimen number. Add one free-falling drop of AST Indicator solution to the AST broth tube. Invert to mix. DO NOT VORTEX.
12. If an inoculum density of 0.50 – 0.60 was used, transfer 25 μL of the bacterial suspension from the ID tube into the AST broth tube.
13. Cap the AST tube and invert several times to mix.
14. Wait a few seconds for air bubbles to surface. You can tap the tube gently to aid in eliminating bubbles.
15. Pour the ID tube inoculum into the fill port on ID side of the panel (51-well side). Allow the fluid to traverse down the tracks before moving the panel. If you are using an AST (only) panel, DO NOT inoculate the ID side of the panel. Retain the ID tube for a recommended purity check (see below).
16. Pour the AST broth inoculum into the fill port on AST side of the panel (85-well side). Allow the fluid to traverse down the tracks before moving the panel.
17. Before placing panel closure check for residual droplets of inoculum on the edge of the fill ports. If a droplet is present remove the droplet with absorbent material. The used absorbent material must be decontaminated before discarding.

18. Snap on the panel closure. Make sure that the closure is fully seated.
19. Visually inspect panels to be sure each of the wells is full. Look at both sides of the panel. Make certain that the wells are not overfilled. If any of the wells are unfilled or overfilled, inoculate a new panel.

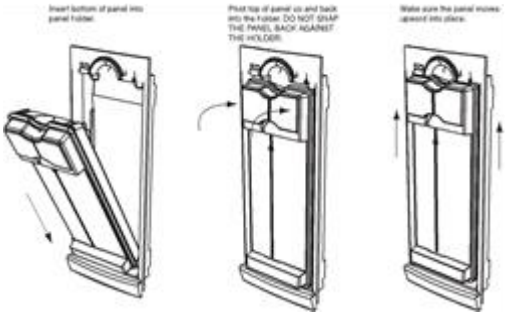
B. Logging in Panels

Log the panel into the instrument as follows:

1. Press the "panel login" soft key.
2. In the Sequence # field, type in or scan the panel's sequence number.
3. In the Accession # field, type in or scan an accession number. Press Tab to advance to the next field (not required if barcode is scanned).
4. The Isolate # field defaults to isolate number 1. Type in the isolate number, or press UP ARROW or DOWN ARROW to increase or decrease the number. Valid isolate numbers are 1 to 20. Press Tab to advance to the next field.
5. If you want to receive special notification (audible alarm and/or automatic printing of a Lab Report) when panel results are obtained (ID only, partial, or complete).
6. If you are only using either the ID or AST portion of a combination panel, press the "tab" key to advance the cursor to the ID or AST checkbox.
7. If the system is not performing the organism identification, you must provide the organism ID for SIR interpretation. (If an AST panel is being tested and you do not enter an organism ID, the panel will go to "Needs Attention" when the instrument completes reading. You will have to provide an organism ID in order for the BDXpert system to interpret MIC results.) Press DOWN ARROW to select the desired organism from the drop down box. Organisms are listed in alphabetical order. You can enter the first few characters of the organism name to jump to that portion of the list quickly. Use UP ARROW or DOWN ARROW to highlight the desired organism. Press the "select" soft key or J to select the organism.
8. Press the "save" soft key to save the information
9. Place the panel in the instrument.

C. Inserting Panels in the Instrument

1. Press the LOAD PANELS key.



2. When the audible signal sounds (and "door unlocked" icon appears), open the instrument door
3. Select a panel holder where there is no panel in place and no LEDs are illuminated.
4. Place the bottom part of the panel in the panel holder.
5. Press downward. Pivot the top of the panel back into the panel holder.
6. Allow the panel to move upward into place.
7. Close the instrument door. If more panels need to be inserted than there are available holders in the current section, wait for a moment for the carousel to rotate to provide additional available holders, and repeat Steps 2 through 7.
8. The system performs an inventory scan to locate any newly inserted panels and reads the barcodes of these panels

D. To access Panel Results

1.  Press the "panel results" soft key from the main status screen or scan an existing accession with the handheld barcode scanner from the main status screen; or scan an existing panel barcode with either scanner from the Panel Login or Main Status screen
2. Panel Results soft keys

"save"  "clear"

E. Adding/modifying ID results

The system enables you to enter an organism ID manually, or to override the organism identified by the instrument. In addition, in some cases, the system will not be able to make a single identification determination based on panel results. To perform the procedure follow the following steps.

1. From the Panel Results display, press the "tab" soft key to advance to the Final ID field.
2. Press the DOWN ARROW key to drop down the listing of organisms.
3. Type the first few letters of the organism name to jump to that alphabetical part of the organism listing.
4. Use the UP ARROW or DOWN ARROW to highlight the desired organism.
5. Press the J or s key, or the "select" soft key to select the highlighted organism

F. Modifying AST results

There may be times when you wish to modify the Final SIR results for a panel. Note that you cannot modify any Final SIR results if there are manual BDXpert rules pending. You must first accept or reject any pending rules, which allows the system to perform its final results processing. After the final processing is complete, you will be able to modify SIR results manually if desired. To modify the Final SIR results:

1. From the Panel Results display, press the "tab" soft key to advance to the Final SIR field for the desired antimicrobial.

2. Press the DOWN ARROW to toggle the field entry among the following results:

S (susceptible)**,** I (intermediate)**,** R (resistant)**,** X = Invalid, cannot interpret

Quality control

Quality Control testing is recommended for each lot of panels. The QC Lot Support feature can

Facilitate QC panel tracking and testing. If the QC Lot Support feature is enabled, then the panel lot number must be defined prior to logging in QC panels.

1. Inoculate a panel with one of the organisms listed in the package insert.

NOTE: All microbial cultures, including QC organisms, are potentially infectious and should be treated with universal precautions. QC organisms are prepared for panel inoculation as specified in procedure Section Preparing Panels, immediately preceding this section.

2. Log the panel in as a QC panel as follows:
    a. Press the "panel login" soft key.
    b. Press the "QC panel" soft key.
    c. In the Sequence # field, type in or scan the panel's sequence number.
    d. If desired, in the Accession # field, type in or scan an accession number. Press T to advance to the next field.
    e. If a combination panel is being used, both the ID and AST checkboxes are automatically checked (enabled).
    f. Type in the isolate number, or press UP ARROW or DOWN ARROW to increase or decrease the number. Valid isolate numbers are 1 to 20.
    g. In the Test Strain field, press DOWN ARROW to drop down the QC organism selection box. See UP ARROW or DOWN ARROW to highlight the desired QC organism. Press J to select the organism. Press T to advance to the next field. (If the QC Lot Support feature is enabled, only the predefined, required test strains appear.)
    h. In the ID field, enter the identification for the technologist performing the QC test. Up to 3 alphanumeric characters are accepted. Press T to advance to the next field. The following two fields do not appear when Epicenter communications is enabled.
    i. In the Panel Lot # field, type in or scan the panel's lot number. Press Tab to advance to the next field. (If the QC Lot Support feature is enabled, this field is completed automatically when the Sequence # barcode is scanned).
    j. In the first Panel Lot # Expiration Date field, press UP ARROW or DOWN ARROW to enter the expiration day, month, or year (depending on your configuration). Press T to advance to the next date field and press UP ARROW or DOWN ARROW to enter the expiration day, month, or year. Press T to advance to the next date field, and press UP ARROW or DOWN ARROW to enter the final portion of the date. (If the QC Lot

Support feature is enabled, this field is completed automatically when the Sequence # barcode is scanned.)

k. If desired, in the ID Broth Lot # field, type in or scan the broth lot number. Lot numbers must be 7 numeric digits. Press T to advance to the next field.

l. If desired, in the first ID Broth Lot # Expiration Date field, press UP ARROW or DOWN ARROW to enter the expiration day, month, or year (depending on your configuration). Press T to advance to the next date field and press UP ARROW or DOWN ARROW to enter the expiration day, month, or year. Press T to advance to the next date field, and press UP ARROW or DOWN ARROW to enter the final portion of the date.

m. If desired, in the AST Broth Lot # field, type in or scan the broth lot number. Press T to advance to the next field.

n. If desired, in the first AST Broth Lot # Expiration Date field, press UP ARROW or DOWN ARROW to enter the expiration day, month, or year (depending on your configuration). Press T to advance to the next date field and press UP ARROW or DOWN ARROW to enter the expiration day, month, or year. Press T to advance to the next date field, and press UP ARROW or DOWN ARROW to enter the final portion of the date.

o. If desired, in the Indicator Lot # field, type in or scan the broth lot number. Lot numbers must be 7 numeric digits. Press T to advance to the next field.

p. If desired, in the first Indicator Lot # Expiration Date field, press UP ARROW or DOWN ARROW to enter the expiration day, month, or year (depending on your configuration). Press T to advance to the next date field and press UP ARROW or DOWN ARROW to enter the expiration day, month, or year. Press T to advance to the next date field, and press UP ARROW or DOWN ARROW to enter the final portion of the date.

q. Press the “save” soft key to save the information.

3.Place the panel in the instrument.

4. When panel testing is complete, review the results for accuracy in the panel results display (42).

III. Gram negative bacilli suspected of beta-lactamase production

 Yes No

IV. Isolate Handling and Storage

Suspected β-lactamase producing gram negative bacilli isolate was sub cultured on 5% sheep blood agar (Oxoid Ltd, UK) to get fresh colonies for phenotypic analysis of ESBL, Carbapenemase and AmpC β-lactamase. After the isolate was analyzed it was stored at -80^{o}C using Tryptose Soy Broth containing 20% Glycerol for future reference or for further analysis.

1. Procedure for Performing the Disk Diffusion Test Inoculum Preparation

 At least three to five pure colonies of the same morphological type was selected from Blood or MacConkey agar plate. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of normal saline.

 The turbidity of the broth culture was adjusted with that of the 0.5 McFarland standards.

Inoculation of Test Plates

 Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level.

 The dried surface of a Mueller Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface.

 The lid may be left ajar for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

NOTE: Extremes in inoculum density must be avoided. Never use unstandardized inoculum for streaking plates.

Application of Disks to Inoculated Agar Plates

 The predetermined antimicrobial disks is dispensed onto the surface of the inoculated agar plate. Each disk must be pressed down to ensure complete contact with the agar surface.

 The plates are inverted and placed in an incubator set to 37ºC within 15 minutes after the disks are applied.

Reading Plates and Interpreting Results

 After 16 to 18 hours of incubation, each plate was examined. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disk. Zones are measured to the nearest whole millimeter, using sliding calipers which is held on the back of the inverted plate.

2. Phenotypic ESBL, Carbapenemase and AmpC detection

I. Phenotypic ESBL Confirmation with Combination Disc Diffusion test (CDDT)

A disc of ceftazidime (30 μg) and cefotaxime (30 μg) alone and ceftazidime + clavulanic acid (30 μg/10 μg) and cefotaxime (30 μg) + clavulanic acid (30 μg/10 μg) was placed at a distance of 25 mm, center to center, on a MHA plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards and incubated overnight (18 – 24 hrs) at 37°C. An increase in the inhibition zone diameter of >5 mm for a combination disc versus ceftazidime or cefotaxime disc alone was confirmed ESBL production.

II. Carbapenemase Detection - Modified Hodge test (MHT)

Carbapenemase production is detected by the MHT when the test isolate produces the enzyme and allows growth of a carbapenem susceptible strain (*E. coli* ATCC 25922) towards a carbapenem disk. The result is a characteristic cloverleaf-like indentation.

Procedure

1. Prepare a 0.5 McFarland dilution of the *E. coli* ATCC 25922 in 5 ml of broth or saline.

2. Dilute 1:10 by adding 0.5 ml of the 0.5 McFarland to 4.5 ml of MHB or saline.

3. Streak a lawn of the 1:10 dilution of *E. coli* ATCC 25922 to a Mueller Hinton agar plate and allow to dry 3–5 minutes.

4. Place a 10 μg meropenem or ertapenem susceptibility disk in the center of the test area.

5. In a straight line, streak test organism from the edge of the disk to the edge of the plate. Up to four organisms can be tested on the same plate with one drug.

6. Incubate overnight at 35OC ± 2OC in ambient air for 16–24 hours

7. After 16–24 hours of incubation, examine the plate for a clover leaf-type indentation at the intersection of the test organism and the *E. coli* 25922, within the zone of inhibition of the carbapenem susceptibility disk.

- **MHT Positive** test has a clover leaf-like indentation of the *E. coli* 25922 growing along the test organism growth streak within the disk diffusion zone.

- **MHT Negative** test has no growth of the *E. coli* 25922 along the test organism growth streak within the disc diffusion.

III. Characterization of carbapenemases- Combined disk test

Four cartridges of tablets containing 10 µg Meropenem (diffusible amount) alone and in combination with inhibitors of different β-lactamases. Inhibitors are added to differentiate between isolates with and without resistance mechanisms (see explanation below). In addition, the KPC/MBL and OXA-48 Confirm Kit (98015) also contains one cartridge of 30 µg Temocillin tablets to detect OXA-48 or similar producing isolates.

Procedure

1. Using a fresh, pure culture prepare a suspension of the organism to be tested equivalent to McFarland 0.5.
2. Using a sterile swap or Drigalski spatula spread the suspension uniformly over the entire area of a Mueller Hinton susceptibility agar plate.
3. Using a single tablet dispenser, place one of each tablet on the inoculated agar plate, ensuring sufficient space between individual tablets to allow for proper measurement of inhibition zones.
4. Incubate at 35±1°C for 18±2 hours (overnight).
5. Measure and record the diameter of the inhibition zones. No zone around a tablet corresponds to a 9 mm inhibition zone.
6. Compare the zone of inhibition of the Meropenem 10 µg tablet to the zones of inhibition of each of the Meropenem + inhibitor tablets. If all zones are within 3mm of each other, record the organism as neither expressing KPC nor MBL activity.

IV. AmpC beta lactamase detection - Combined disk test

Tablets are used for in vitro identification of microbial resistance mechanisms by the agar tablet/disc diffusion method, in order to confirm the mechanism by which the organism has gained resistance to specific antimicrobial agents. This Kit consists of four cartridges of disk diffusion tablets: one cartridge of tablets with Cefotaxime, one with Ceftazidime and two cartridges of the cephalosporins combined with Cloxacillin (AmpC inhibitor). If an organism is suspected of AmpC activity, it can be shown by a difference in the inhibition zone of the cephalosporin alone and in combination with the inhibitor.

Procedure

1. Using a fresh, pure culture prepare a suspension of the organism to be tested equivalent to McFarland 0.5

2. Using a sterile swap spread the suspension uniformly over the entire area of a Mueller Hinton susceptibility agar plate.

3. Using a single tablet dispenser, place one of each tablet on the inoculated agar plate, ensuring sufficient space between individual tablets to allow for proper measurement of inhibition zones. Notice that more than one Confirm Kit can be tested on the same plate.

4. Incubate at 35±1°C for 18±2 hours (overnight)

5. Measure and record the diameter of the inhibition zone. No zone around a tablet corresponds to a 9 mm inhibition zone.

6. Measure the inhibition zones around Cefotaxime 30 µg + Cloxacillin (CTXCX) and Ceftazidime 30 µg + Cloxacillin (CAZCX), and compare with the respective zones around Cefotaxime 30 µg (CTX30) and Ceftazidime 30 µg (CAZ30). If one or both of the combination discs show zones ≥ 5mm than the single discs, the organism is demonstrating AmpC activity.

Annex III. Data collection form for gram negative isolates

1. Isolate identification number:_____
2. Age _____ Sex _____
3. Identification number of the isolate for the study:_____
4. Type of specimen:_____
5. Health facilities : A. Government B. Private
6. Isolated gram negative bacteria:
    - *A. Escherichia coli*
    - *B. Klebsiella pneumoniae,*
    - *C. Klebsiella oxytoca*
    - *D. Proteus mirabilis*
    - *E. Proteus vulgaris*
    - *F. Enterobacter cloacae*
    - *G. Enterobacter aerogenes*
    - *H. Citrobacter freundii*
    - *I. Serratia*
    - *J. Providencia*
    - *K. Salmonella*
    - *L. Shigella*
    - *M. Pseudomonas aeroginosa*
    - *N. Acinetobacter Baumanii*
    - *O. Other*----------
7. Result of AST pattern of the isolates at ICL:

S.No	Antimicrobial Agent	MIC			Result of AST (S/I/R)/(MIC)	Comments
		S	I	R		
1.	Ampicilin	≤ 8	16	≥32		
2.	Gentamicin	≤ 4	8	≥16		
3.	Cefepime	≤2	-	≥ 16		
4.	Amikacin	≤16	32	≥64		
5.	Amoxicillin+clavulanic acid	≤8/4	16/8	≥32/16		
6.	Cefuroxime	≤ 8	16	≥32		
7.	Cefotaxime	≤1	2	≥4		
8.	Ceftriaxone	≤1	2	≥4		
9.	Cefoxitin	≤8	16	≥32		
10.	Ceftazidime	≤4	8	≥16		
11.	Imipenem	≤1	2	≥4		
12.	Ertapenem	≤0.5	1	≥2		
13.	Meropenem	≤1	2	≥4		
14.	Ciprofloxacin	≤1	2	≥4		
15.	Trimethoprim+Sulfamethoxazole	≤2/38	-	≥4/76		
16.	Pipracillin-Tazobactam	≤16/4	32/4–64/4	≥128/4		
17.	Aztreonam	≤4	8	≥16		

8. Screening test for ESBL, AmpC, Carbapenem resistant gram negative bacteria on phoenix system:

A. ESBL B. Potential carbapenemase producer C. Potential AmpC β-lactamase producer

Annex IV. Declaration

Declaration

I the undersigned candidate, declare that this M.Sc. thesis is my original work and has not been presented for a degree in this or any other university and that all sources of materials used for the thesis have been duly acknowledged.

M.Sc. Candidate: Saba G/Michael Tekele (B.Sc.)

Signature:_____

Date of submission:_____

This thesis has been submitted with our approval as advisors.

Advisor: Mr. Samuel Kinde **(MSc, PhD candidate)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: Mr. Kassu Desta **(MSc, PhD candidate)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.