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Extended-spectrum Beta-lactamase producing *Enterobacteriaceae* in clinical specimens at selected Microbiology Laboratories, Addis Ababa, Ethiopia

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This is to certify that the thesis prepared by Dejenie ShiferawTeklu, entitled: **Extended-spectrum beta-lactamase producing *Enterobacteriaceae* in clinical specimens at selected Microbiology Laboratories, Addis Ababa, Ethiopia** and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology Specialty) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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List of Abbreviations

AMR	Antimicrobial Resistance
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
CDT	Combination disk test
CLSI	Clinical and Laboratory Standards Institute
DDST	Double disk synergy test
DRER	Department of research and ethics review committee
EPHI	Ethiopian Public Health Institute
ESBLs	Extended spectrum β-lactamases
ESBL-E	Extended spectrum β-lactamases *Enterobacteriaceae*
MAC	MacConkey agar
MBL	Metallo beta-lactamases
MDR	Multidrug Resistant
MHA	Müeller Hinton agar
ICL	International Clinical Laboratories
SHV	Sulfhydryl variable
SMART	Study for Monitoring Antimicrobial Resistance Trends
SSA	Sub-Saharan Africa
TEM	Temoniera (name of a patient)
TSB	Tryptose Soya Broth
TASH	Tikur Anbessa Specialized Hospital
YHMC	Yekatit 12 Hospital Medical College
WHO	World Health Organization

Operational Definitions

Multi-drug Resistance (MDR): when a bacterium was simultaneously non susceptible to three or more antibiotics of different classes.

Extended spectrum bata-lactamase producing *Enterobacteriaceae*: is a bacterium that produces enzymes against penicillins and 1st, 2nd, 3rd generation cephalosporin to become resistant but not cephamycins (cefoxitin) or carbapenems (e.g meropenem).

Non susceptible: is result for resistance and intermediate antibiotics susceptibility.

Non-ESBLs suspicious *Enterobacteriaceae*: are bacteria that are ESBLs screening negative.

ABSTRACT

Background: The current estimated number of deaths worldwide due to drug-resistant infections is about 700,000 each year. The emergence and spread of Extended-spectrum beta-lactamases (ESBLs) producing *Enterobacteriaceae* becomes one of the serious health problems globally.

Objective: To determine the magnitude of Extended-spectrum beta-lactamase producing *Enterobacteriaceae* (ESBLs-E) in different clinical specimens at selected microbiology laboratories, Addis Ababa, Ethiopia.

Methods: A cross-sectional study was conducted from January 1 to May 30, 2017. A total of 426 *Enterobacteriaceae* isolates were collected from four bacteriology laboratories using tryptose soya broth with 20% glycerol. Fresh colonies of the isolates were recovered using MacConkey and 5% sheep blood agar plate. Antimicrobial susceptibility testing was performed on Muller Hinton agar. All *Enterobacteriaceae* were screened for ESBLs production using cefotaxime and ceftazidime as per Clinical and Laboratory Standards Institute (CLSI) guideline. Each ESBLs specious *Enterobacteriaceae* were confirmed by combination disk test (CDT) and double disk synergy test (DDST). The sensitivity, specificity, positive and negative predictive value of DDST was calculated. Data was entered and analyzed using SPSS version 20.

Results: The most frequent *Enterobacteriaceae* were *E. coli* 228 (53.5%) and *K. pneumoniae* 103 (24.1 %). The magnitude of ESBLs-E was 246 (57.7%) by CDT and 224 (52.6%) by DDST. The highest frequencies of ESBLs-E were observed in blood specimen (84.4%) ($P < 0.05$) and the highest ESBLs production was observed in *K. pneumoniae* (85.4%). Highest resistance level was seen to sulfamethoxazole-trimethoprim (77.0%), augumentin (71.6%), cefotaxime (62.2%), cefepime (60.3) and ceftazidime (60.8%). The resistance to meropenem, amikacin and cefoxitin were 5.2%, 13.8% and 25.1% respectively. The overall magnitude of multi-drug resistance (MDR) level was 68.3%. Of ESBLs-E, 96.3% were MDR ($P < 0.05$). The sensitivity, specificity, PPV and NPV of DDST was 91.1%, 100%, 100% and 46.3% respectively.

Conclusion: There was a high magnitude of ESBLs producing *Enterobacteriaceae* and MDR. Hence, the control of ESBLs should be a high priority. The better antibiotics for ESBLs producing *Enterobacteriaceae* were meropenem and amikacin. ESBLs detection by DDST routinely can help for infection control and timely treatment of a patient with best antibiotics.

Keywords: Extended-spectrum beta-lactamase, Multi-drug Resistance, *Enterobacteriaceae*, Clinical specimens, Combination disk test, Double disk synergy test, Ethiopia

1. INTRODUCTION

1.1 Background

Enterobacteriaceae such as *Escherichia spps., Klebsiella spps. and Enterobacter spps.,* are Gram-negative, facultative anaerobes, and non-sporing bacilli (1). These bacteria have become one of the most important causes of nosocomial and community-acquired infections such as urinary tract infection, respiratory tract infection, gastro intestinal tract infection, wound infection and blood stream infection. Increasing rates of antimicrobial resistance have become a worldwide problem predominantly caused by gram-negative bacteria, especially the *Enterobacteriaceae* family (2,3).

The most prescribed antibiotics to treat infections caused by *Enterobacteriaceae* are; beta-lactams drugs (e.g penicillins, cephalosporins, and monobactams) fluoroquinolones (e.g. ciprofloxacin) and aminoglycosides (e.g. gentamicin). The widespread use of beta-lactam antibiotics has caused the expansion of resistant *Enterobacteriaceae*. The predominant resistance mechanism to beta-lactams is the production of beta-lactamases that inactivate beta-lactam antibiotics and continues to be the prominent cause of β-lactam antibiotics resistance among *Enterobacteriaceae* worldwide (4,5). One of the major types of beta-lactamase enzyme produced by *Enterobacteriaceae* is extended-spectrum beta-lactamases (ESBLs). ESBLs producing *Enterobacteriaceae* are important members of antibiotic-resistant bacteria that cause hospital and community-acquired infections (6).

ESBLs are enzymes that hydrolyse penicillins, 1st, 2nd and 3rd generation cephalosporins and aztreonam but do no hydrolyse cephamycins (cefoxitin) or carbapenems and are generally inhibited by beta-lactamase inhibitors such as clavulanic acid and sulbactam (7). They belong to Amber's molecular class A and Bush's group 2be functional classification (8,9). ESBLs are derived from their progenitors TEM-1, TEM-1 or SHV-1 beta-lactamases by amino acid substitutions and differ by a few amino acid sequences (point mutation) (8). There are also ESBLs producing *Enterobacteriaceae* species, including CTX-M and OXA-type, which are not closely related to TEM or SHV (2). A worrisome increasing trend has been seen in the development of resistance to extended-spectrum cephalosporins by ESBLs producing

Enterobacteriaceae (2,3,7). ESBLs occur predominantly in *K. pneumoniae* and *E. coli.* In addition, it may also be present in other members of *Enterobacteriaceae*, such as *Enterobacter spps.*, *Proteus spps*, *Citrobacter spps*, *Morganella spps*, *Providencia spps.*, *Salmonella spps.*, and *Serratia spps.* (10,11).

Being plasmid mediated, ESBLs are easily transmitted among members of *Enterobacteriaceae* and other bacteria thus facilitating the dissemination of resistance gene among bacteria not only to β-lactams but also to other commonly used non-beta-lactam antibiotics families. For this reason, in addition to 1st to 3rd generation cephalosporin, ESBLs producing *Enterobacteriaceae* are often resistant to other antibiotic families such as fluoroquinolones, aminoglycosides and sulphonamides (12,13). Consequently, many patients need the 'last resort' antibiotics treatment like carbapenems drugs (2,14). Again the use of carbapenems has led to the rapid selection of carbapenem-resistant *Enterobacteriaceae* (15). Only a few antibiotic like carbapenems, colistin, tigecycline are available to treat infection caused by ESBLs producing bacteria, although their in vivo efficacy and/or toxicity profile is not well known (16,17).

Laboratory detection of ESBLs involves two steps. The first is a screening test with an indicator cephalosporin which looks for resistance or reduced susceptibility. The CLSI has proposed disk diffusion dilution methods for screening of ESBLs producing *K. pneumoniae* and *K. oxytoca, E. coli* and *P. mirabilis*. Growth at or above the screening antibiotic susceptibility cut off value is suspicious of ESBLs production and is an indication for the bacteria to be tested by a phenotypic confirmatory test (18).

The second is confirmatory test that look for synergy between cephalosporin and clavulinic acid, which differentiate isolates with ESBLs enzyme from those that are resistant for other reasons. Several phenotypic detection methods have been developed to confirm ESBLs production by *Enterobacteriaceae* such as Combination Disks Test (CDT), Double-Disk Synergy Test (DDST), Broth Microdilution Assay, Three-Dimensional Test, E-Test, Inhibitor-potentiated disk-diffusion test (19). CLSI recommended CDT and Jarlier and his colleague's DDST are the most commonly used methods as a phenotypic confirmatory test for ESBLs producing *Enterobacteriaceae* (18,20).

1.2 Statement of the Problem

The global emergence and spread of antibiotic-resistant bacteria have been threatening the ability to treat common infectious diseases and result in prolonged illness, disability, and death. Currently, it has been happening in every region of the world and has the potential to affect anyone, of any age, in any country. In fact, no new major types of antibiotics have been developed in over 30 years (21). *Enterobacteriaceae* families are responsible for a large proportion of serious, life-threatening infections and resistance to multiple antibiotics. It is estimated that, by 2050, the global cumulative cost on antibiotic resistance will reach 100 trillion USD (22).

Extended-spectrum beta-lactamase (ESBLs) producing pathogenic *Enterobacteriaceae* cause a serious antibiotic management problem, as the enzyme-encoding genes are easily transferred from one organism to the other via plasmids (23). Due to the fact that ESBLs producing *Enterobacteriaceae* are also resistant to other antibiotics families, advanced procedures like surgeries, cancer treatment, and organ transplantation could become increasingly risky (24). Inability to treat these patients would increase morbidity and mortality. This problem is more pronounced in areas where there is no adequate infection control program, periodic surveillance and multidrug resistant bacteria detection laboratory facility (25).

In the members of *Enterobacteriaceae* family, the production of plasmid-mediated ESBLs have emerged as the main mechanism of resistance to beta-lactam antibiotics, the drugs that account for about 50% of world antibiotic consumption (26). The effect of antibiotics resistance is not only on the individual patient but also on the entire health systems. Patients who are infected with drug resistant bacteria require more complicated treatment, take longer to recover, and are more likely suffer treatment failure and death. This will result in devastating financial, social, and psychological effects in the family. Furthermore, AMR also imposes enormous financial burdens on society as a whole; patients with AMR-related illnesses may miss work for longer periods, which results in loss of productivity and income. For example, the health care costs and lost productivity cost is estimated to $55-70 billion per year in USA and €1.5 billion per year in Europe (21,22,24).

More than 50% of medicines are prescribed, dispensed, or sold inappropriately worldwide. Due to this the prevalence of AMR bacteria are increasing. The current estimated number of deaths worldwide due to drug-resistant infections is approximately 700,000 each year. This figure is likely to reach as high as 10 million per year by 2050 if AMR remains unchecked. CDC estimates that antibiotic resistance is responsible for more than 2 million infections and 23,000 deaths each year in the United States. In Europe, a similar picture is developing, with an estimated 25,000 deaths attributable to antibiotic-resistant infections. In 2014, nearly 25% of hospital-acquired infections in the United States were caused by antibiotic-resistant bacteria as estimated by the US CDC as public health threats (21,22,27,28).

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. This condition is aggravated by lack of good hygiene, lack of safe water, civil conflicts, lack of strong infection control strategy and increasing numbers of immune-compromised people (29). In East Africa, blood stream infection caused by gram negative bacteria exhibited relatively high levels of resistance to antibiotics commonly used (50% - 100% to ampicillin and cotrimoxazole). Furthermore, significant levels of resistance to cefrtiaxone and gentamicin (20% - 47%) was also reported (30).

Current knowledge of the magnitude of ESBLs producing *Enterobacteriaceae* is important to understand their epidemiology, the disease burden and to implement hospital infection control strategy to prevent the spread of these bacteria. Although extended spectrum beta-lactam resistant *Enterobacteriaceae* cause significant and devastating public health problems, little is known about the magnitude of ESBLs producing *Enterobacteriaceae* in Ethiopia. Previously, only few studies have been conducted in Ethiopia for ESBLs detection which determined ESBLs producing *Enterobacteriaceae* prevalence from few *Enterobacteriaceae* isolates. Furthermore, to the best of our knowledge, most microbiology laboratories in Ethiopia do not perform ESBLs detection tests both for the diagnostic and for infection control or surveillance purpose. Hence, this study aimed to determine the magnitude of ESBLs producing *Enterobacteriaceae* and their antimicrobial resistance pattern from clinical specimens in selected microbiology laboratories, Addis Ababa, Ethiopia.

1.3 Significance of the Study

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- The current information generated from this study can be used by different responsible bodies such as microbiologists and infectious disease specialists for the control and containment of ESBLs producing *Enterobacteriaceae*.
- The study has also provided information about antimicrobial resistance pattern of ESBLs and non-ESBLs producing *Enterobacteriaceae* with their multidrug resistance level. This can help physicians for better antimicrobial prescription to *Enterobacteriaceae* and other responsible bodies to control further occurrence and spread of this group of bacteria.
- The result obtained from this study can be used for recommendation and the implementation of ESBLs screening and confirmatory test, because the early detection of these bacteria is important to prevent treatment failure and to control nosocomial infection and outbreaks.
- The study has generated information about usefulness of double disk synergy test to detect and confirm ESBLs producing *Enterobacteriaceae* in resource limiting settings by comparing it with combination disk method. Hence, policy makers can use this information for the recommendation and implementation of this simple ESBLs screening and confirmatory method in Ethiopian microbiology laboratories because the early detection of these bacteria is important to prevent treatment failure, to control nosocomial infection and outbreaks.
- The detection of ESBLs producing *Enterobacteriaceae* provides supplementary baseline information to plan for infection control and surveillance.

2 LITERATURE REVIEW

2.1 *Enterobacteriaceae*

Enterobacteriaceae are family bacteria that are rod-shaped, Gram-negative, non-spore forming, facultative anaerobes and ferment different carbohydrates to obtain carbon. They are heterogeneous bacterial family consisting of more than 30 genera and 150 species and subspecies. Although many *Enterobacteriaceae* are widespread in the environment, most of them are components of the gastrointestinal flora of humans and animals and may cause intestinal and extra-intestinal infections. They can cause different types of infections such as urinary tract infections (UTIs) (the most common), pneumonia, wound infections and infections of the bloodstream and central nervous system. Some genera are common causes of intestinal infections such as enteritis and diarrhoea. Medically important species of *Enterobacteriaceae* are *Escherichia spps., Klebsiella spps., Enterobacter spps., Citrobacter spps., Proteus spps., Providencia spps., Serratia spps., Salmonella spps., and Shigella spps.* (1).

E. coli is the most abundant *Enterobacteriaceae* of the human intestinal microflora. The normal flora *E. coli* strains rarely cause disease in humans, except in immunosuppressed patients or when the normal gastrointestinal barriers are out of order. A limited number of pathogenic *E. coli* clones have gained specific virulence factors which enable them to cause urinary tract infections, septicaemia, menigitis and diarrheal disease both in immunocompromised patients and in immunocompetent individuals (31).

K. pneumoniae typically causes opportunistic infections of the urinary tract, in wounds and soft tissue and in the blood stream (32). Infections caused by *K. pneumoniae* are often observed in hospital area and are associated with high morbidity and mortality rates in infants and the elderly (33,34). From the many factors contribute to the pathogenesis of *K. pneumoniae*, the prominent capsule and lipopolysaccharides are the main for the ability of this species to form biofilm and to colonies an organ (35).

2.2 Beta-lactam Antibiotics

Beta-lactam antibiotics are among the most commonly prescribed drugs (36), grouped together based upon a shared structural feature, the beta-lactam ring. They are antibiotics are generally bactericidal against organisms that they inhibit. Beta-lactam antibiotics include: Penicillins, Cephalosporins, Cephamycins, Carbapenems and Beta-lactam inhibitors. Cephalosporins are divided into 1st to 5th generation according to their antibacterial activity (Table 1). They differ in their antimicrobial spectrum, beta-lactamase stability, absorption, metabolism, stability and side-effects. First-generation members have narrowed or limited activity when compared with third-generation, fourth-generation or fifth-generation broader spectrum cephalosporins (37).

Table 2.1: Major group of cephalosporins according to their antimicrobial activity

First-generation	Second-generation	Third-generation	Fourth-generation	Fifth-generation
Cephalothin	Cefamandole	Cefotaxime	Cefepime	Ceftobiprole
Cephapirin	Cefuroxime	Ceftizoxime	Cefpirome	Ceftaroline
Cefazolin	Cefonicid	Ceftriaxone		
Cephalexin[a]	Ceforanid	Ceftazidime		
Cephradine[a]	Cefoxitin[b]	Cefoperazone		
Cefadroxil[a]	Cefmetazole[b]	Cefixime[a]		
	Cefminox[b]	Ceftibuten[a]		
	Cefotetan[b]	Cefdinir[a]		

[a]Oral cephalosporins; all the others are parental cephalosporins. [b]Besides being cephamycins (chemical classification), they are usually included in the microbiological classification as second-generation cephems.

2.3 Mechanism of antibiotics action and resistance

Generally, there are five categories of mechanisms of antibiotics action (Fig. 5). These are inhibition of cell wall synthesis; impairment cytoplasmic membrane; inhibition of nucleic acid synthesis; inhibition of protein synthesis; and metabolic antagonist action. In the other side, there are four basic mechanisms (Fig. 5) by which resistance to drug may occur in bacteria: alteration of the antimicrobial target that can be due to the complete loss of affinity or simple reduction of it; reduction in the amount of the antimicrobial that reaches the target by entrance reduction caused by a decrease permeability due to porin mutation or by an exit increase caused by the pumping out by an efflux transporter; the presence of an enzymatic mechanism that totally or partially destroys the antimicrobial molecules; and the development of an alternative metabolic pathway involving precursors (38–40).

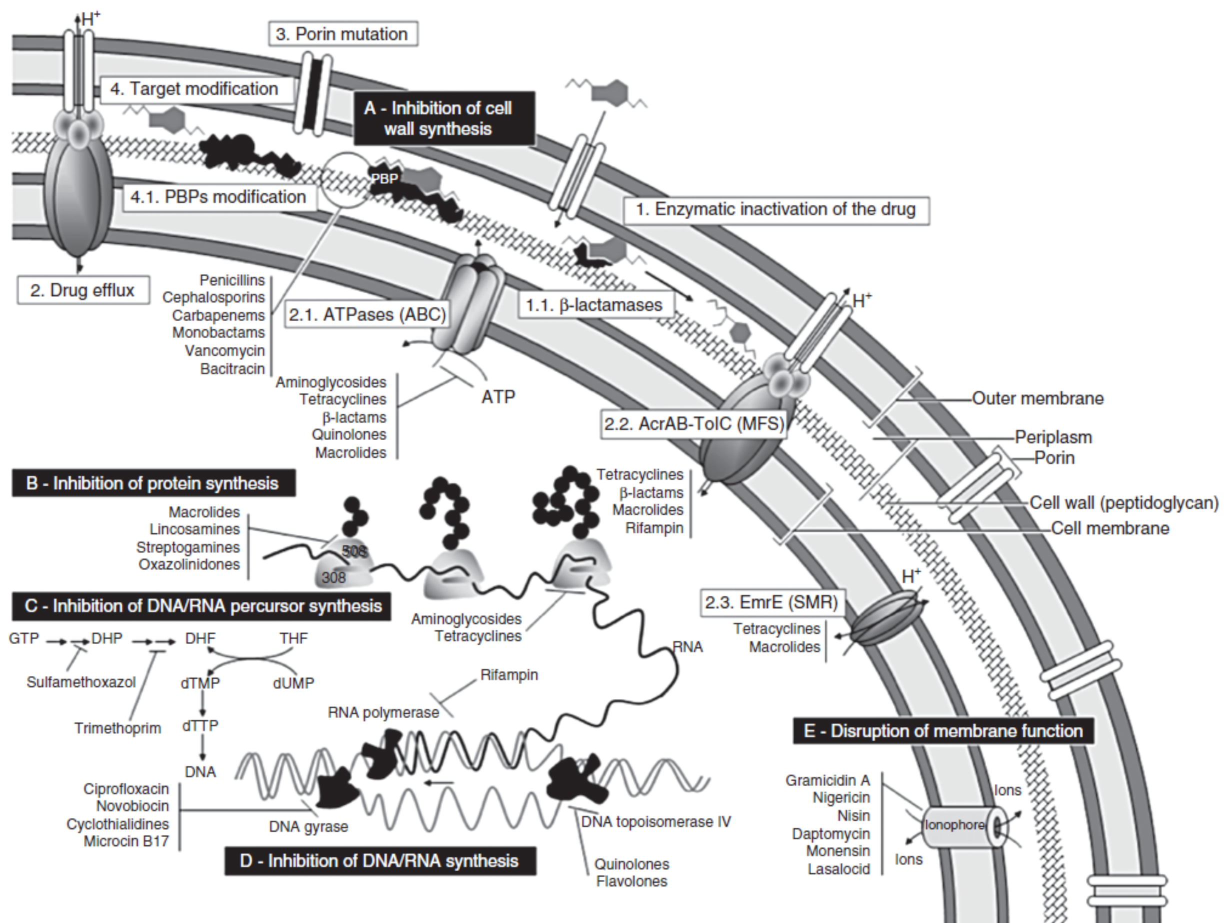


Figure 2.1: Mechanisms of antimicrobial action and resistance in Gram-negative organisms. This picture represents a Gram-negative bacteria cell. Black boxes represent mechanisms of drug action and white boxes represent mechanisms of resistance. Below each box there are several examples of drugs presenting those types of mechanisms. The main mechanisms of antimicrobial action can be divided into five major classes. (A) Those who act in the cell wall synthesis; (B) those who act in the protein translation; (C) those who act in metabolic precursor biosynthesis; (D) those who act in the molecular genetics processes (replication, transcription); and (E) those who disrupt membrane function and permeability. Some of the mechanisms of resistance are represented here by numbers. (1) Enzymatic inactivation of the drug by the presence of b-lactamases (1.1); (2) presence of an enhanced efflux pump, whether it is by an active transport system involving ATPases (2.1) or rather if it is driven by proton motive force (2.2, 2. 3); (3) porin mutation obstructing the drug entrance; and (4) target modification of the drug, such as the mutation in the penicillin binding proteins (PBPs) (37).

2.4 Mechanism of Beta-lactam antibiotics action and resistance

The mechanism of bacterial cell killing of beta-lactam antibiotics is an indirect consequence of the inhibition of bacterial cell wall synthesis. Beta-lactam antibiotics are characterized by its four-membered, nitrogen-containing beta-lactam ring at the core of their structure, which is key to the mode of action. Beta lactam antibiotics target the penicillin-binding proteins or PBPs - a group of enzymes found anchored in the cell membranes, which are involved in the cross-linking of the bacterial cell wall. The beta-lactam ring portion of this group of antibiotics binds to these different PBPs, rendering them unable to perform their role in cell wall synthesis. This then leads to death of the bacterial cell due to osmotic instability or autolysis (39,40).

2.5 Beta-lactamases and Extended Spectrum Beta-lactamase

The beta-lactamases are the broad name of enzymes that hydrolyse the beta-lactam ring by adding a water molecule to the common beta-lactam bond, and this inactivates the beta-lactam antibiotic from penicillin to carbapenems. Since beta-lactam was the first antibiotic to be described, resistance to beta-lactam antibiotics is the first to be understood (41). The most effective way for bacteria to neutralize these antibiotics has been by producing beta-lactamases, enzymes that inactivate the drugs by hydrolyzing the beta-lactam ring. Based on the sequence analysis, beta-lactamases and the PBPs are believed to diverge from a common ancestor (42).

Two classification schemes exist for beta-lactamases. The first one is Ambler structural classification (classes A-D), which is based on amino acid sequence homology (8). The second is the Bush–Jacoby-Medeiros functional classification (groups 1-4), that is based on substrate and susceptibility to the inhibitor clavulanic acid. ESBLs fall into Ambler´s class A and subgroup 2be. The 2be designation means that these enzymes are derived from group 2b beta-lactamases (TEM-1, TEM-2, and SHV-1); the "e" of 2be denotes that the beta-lactamases have an extended spectrum (9). A complete update and overview of the individual beta-lactamases is available at www.lahey.org/studies.

The three main types of ESBLs described are TEM, SHV and CTX-M. The earliest ESBLs , first identified in the 1980s, were mutants of the plasmid-borne parent enzymes TEM-1, TEM-2 and SHV-1 beta-lactamases (43). Over time, these enzymes have undergone amino acid substitutions,

which resulted in more than 300 currently known TEM and SHV ESBL variants. The ESBLs derived from TEM and SHV could differ from their progenitors by only one amino acid. This change was critical and had a profound effect on enzymatic activity, leading to hydrolysis of third-generation cephalosporins and aztreonam (7). A massive shift in the distribution of ESBLs has occurred since 2000 with the spread of CTX-M type ESBLs of which five subgroups (groups 1, 2, 8, 9 and 25) are circulating worldwide, both in nosocomial and community settings (44).

2.6 Prevalence Extended Spectrum Beta-lactamase in different country

Extended Spectrum Beta-lactamase (ESBLs) producing *Enterobacteriaceae* are a problem in especially hospitalized patients throughout the world. The prevalence of ESBL among clinical isolates varies greatly worldwide and within geographic areas, and is rapidly changing over time. Multiple global surveillance programs document an expanding distribution with increasing ESBLs prevalence worldwide (45–48).

The Study for Monitoring Antimicrobial Resistance Trends (SMART) by Ian Morrissey and his colleagues (45) had followed resistance patterns of gram-negative bacteria worldwide from 2002 to 2011. Over the course of the SMART study, the five most commonly isolated Gram-negative pathogens from intra-abdominal urinary tract infections were *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *E. cloacae* and *P. mirabilis.* In this surveillance, significant increases in infections by ESBL-producing bacteria were found across all studied continents, with the exception of Africa. More than 40% of clinical isolates from Asia were ESBL producers in 2011. Moreover, Latin America, the Middle East, Africa, Europe, and the South Pacific displayed a prevalence of ESBL of approximately 10%–35% (45).

In 2012, a study from nine US census regions reported ESBLs proportion estimates in the 4 to 12 % range from *Enterobacteriaceae* that were collected from 72 hospitals. In this study, the highest ESBLs producers were seen in *K. pneumoniae* (16.0%), 4.8 to 11.9% for the other species (47). From the European country, the average ESBLs proportion estimates reported in a nationwide hospital survey in Germany for 2012 were in the 10 to 15 % range (49).

A nine years survey in Japan reported ESBLs proportion estimate of 6.3 % in 2003 that increased to 10–20 % in 2011 (50). A Nationwide survey conducted in China that included 30 hospitals

reported over 46 % resistance due to ESBLs (51). In a study conducted in central India from 1044 *Enterobacteriaceae* isolates recovered from various clinical specimens, ESBL production was confirmed in 504 (48.27%) isolates. The majority of ESBL isolates were from urine samples (52.28%) and *E. coli* were the most common ESBL producers (50.14%) followed by *K. pneumoniae* (48.27%). In this study the majority of ESBLs producers (>80%) were sensitive to meropenem, piperacillin-tazobactam, and amikacin. The susceptibility of ESBL-producing urinary isolates to nitrofurantoin and nalidixic acid was 82.5% and 15.16%, respectively indicating co-resistance among ESBL isolates. It was only 50% of ESBLs isolates that were sensitive to gentamicin. Significant resistance was detected against trimethoprim–sulfomethoxazole, fluoroquinolones, and gentamicin (52).

The Middle East countries, in a study conducted among inpatients in Saudi Arabia in 2008, by Tawfik and his colleagues found that 26% of *K. pneumoniae* isolates produced ESBLs (53). Another investigation conducted in the same country in 2004–2005 showed that 10% of clinical urinary *E. coli* isolates from inpatients and 4% of such isolates from outpatients were ESBL producers (54).

Different studies done in different African countries have showed that the prevalence of ESBLs-producing *Enterobacteriaceae* varies between the countries and the type of specimen studied. There is a trend of higher prevalence of ESBL in stool samples than in other specimens and increasing prevalence over time. When we see the study conducted in the two Tunisian Hospitals, in the study periods from 1999–2005 to 2010, ESBLs have increased from 11.7 to 77.8% among *K. pneumonia* in mainly urine, blood, and stool samples (55).

A study conducted in Accra, Ghana at Korle-Bu Teaching Hospital from various clinical specimens, from the total of 300 *Enterobacteriaceae* isolates 49.3% were ESBLs producer using combined-disk method. In the study urinary tract infection was the most abundant source 66.70% (70/105) of ESBLs producers. In addition, ESBLs producing isolates had increased resistance compared with non–ESBLs producers to cotrimoxazole (92.6%, 57.2%), gentamicin (91.2%, 50.6%), amikacin (44.8%, 20.5%), and ciprofloxacin (41.1%, 21.1%), respectively, but all isolates were susceptible to meropenem (56).

The study conducted in Tanzania in 2001–2002 on blood isolates from neonates showed that 25% of the *E. coli* and 17% of the *K. pneumoniae* produced ESBLs (57). In the same country at a tertiary hospital in Mwanza, Tanzania, from 377 clinical isolates the overall prevalence of ESBLs in all Gram-negative bacteria was 29%. From all Gram-negative bacteria, ESBLs prevalence was 64% in *K. pneumoniae* and 24% in *E. coli* (58).

In a study conducted at an orphanage in Mali significant figures were also obtained, where 63% of the adults and 100% of the children were found to carry ESBLs producing *Enterobacteriaceae* that showed extensive co-resistance to other non beta-lactam antibiotics (59). In a study conducted in Egypt from 1999-2000 on *E. coli* isolates collected at five hospitals, it was found that 38% were resistant to third-generation cephalosporins (60). In addition, another investigation conducted in the same country in 2001 showed that 61% of *E. coli* produced ESBLs (61).

The overall pooled ESBL proportion estimate for East African hospitals was 42 %. The East African proportion of ESBLs is greatly higher than averages reported for resource-rich countries like USA and Europe (62). From the study conducted in East Africa Uganda reported 62% ESBLs out of 115 isolate of *E. coli*, *K. pneumoniae*, *P. mirabilis* and others *Enterobacteriaceae* from Urine, blood, wound, CSF specimens using DDST method (63).

Few studies were conducted in Ethiopia on the prevalence of ESBL. The highest prevalence was published from Bahir Dar. By using E-test method they reported 57.6% ESBLs out of 210 *K. pneumoniae* and *E. coli* isolated from blood, urine, pus, ear discharges, wound swab from surgical site infections and cerebrospinal fluid (CSF) specimens (64).

A study conducted in Jimma-Ethiopia using DDST method showed that from 112 isolate of *K. pneumoniae* and *E. coli* isolated from urine, vaginal swab, sputum, pus, eye discharge and blood specimens 38.4% of them are ESBLs. *K. pneumoniae* (70.4%) and *E. coli* 28.2% was the predominant ESBLs producer (65).

Another study conducted in Adama-Ethiopia reported 25% ESBLs out of 63 isolate of *E. coli, Proteus* species, *Klebsiella* species, *E. cloacae* and *Citrobacter* species from different clinical specimens using DDST method. The predominant ESBLs producer was *E. coli* 28.57% (10/35) followed by *Proteus species* 33.3% (3/9) and *Klebsiella species,* 25 (2/8) (66).

A study conducted in Tikur Anbessa Specialized Hospital, Addis Ababa, among children suspected of septicemia and urinary tract infections, reported an overall prevalence of 78.6% ESBLs producing *Enterobacteriaceae*; from these *K. pneumoniae* (84.2%, 16/19), and *E. coli* (100%, 5/5) were the most predominant. In this study DDST was compared against CDT and they found sensitivity, specificity, positive predictive value and negative predictive value of 90.9%, 66.7%, 95.2% and 50% respectively (67).

3 OBJECTIVE OF THE STUDY

3.1 General Objective:

To determine the magnitude of ESBLs producing *Enterobacteriaceae* and antibiotics resistance pattern in different clinical specimens at selected microbiology laboratories in Addis Ababa, Ethiopia.

3.2 Specific Objectives:

- To determine the magnitude of ESBLs producing *Enterobacteriaceae* in different clinical specimens.
- To determine antimicrobial resistance pattern of *Enterobacteriaceae*.
- To determine the magnitude of multidrug resistance *Enterobacteriaceae*.
- To compare Double disk synergy test (DDST) against Combination Disk Test (CDT) for the detection of ESBLs.

4 Hypothesis

The magnitude of ESBLs producing *Enterobacteriaceae* isolates in Addis Ababa is different from previous studies conducted in Ethiopia.

5 MATERIALS AND METHODS

5.1 Study Setting

The study was conducted at Ethiopian Public Health Institute (EPHI) in Clinical Bacteriology laboratory section. The EPHI Clinical Bacteriology Laboratory is a referral laboratory in the country. This laboratory is accredited by Ethiopian National Accreditation office (ENAO). Including the EPHI bacteriology laboratory, the *Enterobacteriaceae* isolates were collected from another three microbiology laboratories found in Addis Ababa namely International Clinical Laboratories (ICL), Tikur Anbessa Specialized Hospital (TASH) and Yekatit 12 Medical College Hospital Microbiology Laboratory (YHMC). Except International Clinical Laboratories which is a private organization the rest laboratories are owned by government.

TASH is the largest and oldest specialized teaching hospital with 500 beds in Addis Ababa, Ethiopia that provides referral service for patients coming from all side of the country. The hospital provides service to outpatients and patients admitted in different wards such as medical, surgical, pediatric, neonatal, obstetric and gynecologic. YHMC is one of the tertiary level referral and teaching hospital in Addis Ababa. The hospital provides health care services with inpatient and outpatient department to community in Addis Ababa, and referral cases from different health center of Addis Ababa and its neighborhood. ICL which is accredited by Joint commission International serves patients who come from Addis Ababa as well as specimens referred from all over the regions of Ethiopia. ICL has 5 patient service centers in Addis Ababa, and 9 in the different regions of Ethiopia which facilitates the specimen referral process to the central laboratory found in Addis Ababa.

5.2 Study Design & Period

A cross-sectional study design was used and the study was conducted from January 1 to May 30, 2017.

5.3 Source Population

The source population was all *Enterobacteriaceae* isolated from different clinical specimens in Addis Ababa, Ethiopia during the study period.

5.4 Study Population

The study population was all *Enterobacteriaceae* isolated in the selected microbiology laboratories that fulfill the inclusion criteria during the study period.

5.5 Inclusion Criteria

All consecutive *Enterobacteriaceae* isolated from different clinical specimens in the selected bacteriology laboratories, Addis Ababa, Ethiopia, during the study period was included.

5.6 Exclusion Criteria

- *Enterobacteriaceae* isolated from stool.
- Improperly labeled isolate
- Contaminated isolate.
- Duplicate *Enterobacteriaceae* from the same patient.

5.7 Sample Size Determination

The sample size was calculated based on a single population proportion formula. The expected prevalence, p=57.6% was taken from Bayeh's Study in Bahir Dar (64).

Sample size calculation formula; $\mathbf{n = z^2 p (1-p)/d^2}$ Where n = sample size, *Z* = Z statistic for a level of confidence (95% level of confidence; z=1.96), *P* = expected prevalence or proportion (*P*= 0.576 and *d* = precision (in proportion of one; if 5%, *d* = 0.0469).

$$n = (1.96)^2 (0.576) (1-0.576) / (0.0469)^2$$

n= 426

During the study period, 426 *Enterobacteriaceae* isolates were collected and all isolates were included in this study to determine the magnitude of ESBLs producing *Enterobacteriaceae*.

5.8 Sampling Method

A purposive sampling technique was applied to select volunteered microbiology laboratories and have microbiology laboratory in Addis Ababa. While a convenient sampling technique was used to collect *Enterobacteriaceae* isolates from different clinical specimens.

5.9 Study Variables

5.9.1 Dependent Variables

- Magnitude of ESBLs producing *Enterobacteriaceae*
- Antibiotic resistance pattern of *Enterobacteriaceae*

5.9.2 Independent Variables

- Age group
- Gender
- Study sites
- Type of specimens

5.10 Data Collection Procedure

The demographic data of patients was recorded using a pre-developed data collection sheet from the request form and record book. Using data collection sheet; the age and sex of the patient, type of specimen, the *Enterobacteriaceae* isolated and the culture method they used for isolation, and the antibiotics susceptibility pattern of the isolate were collected. All of this information was collected by the principal investigator.

5.11 Laboratory Methods

5.11.1 Isolates Collection and Handling

The non duplicate consecutive *Enterobacteriaceae* isolates were collected using Tryptose Soy Broth (TSB) (Oxoid LTD, Basingstoke, Hampshire, England) containing 20% Glycerol and temporarily stored at -20°C or -70°C in the selected laboratories until transported to EPHI clinical bacteriology laboratories. The stored isolates from these laboratories were transported to EPHI Clinical Bacteriological Laboratory using cold box with ice. After the isolate was analyzed it was stored at -70 ^{o}C for future reference or for further analysis.

5.11.2 Culture

The isolates preserved at -70°C were recovered by re-suspension in *Tryptose Soy Broth* (Oxoid LTD, Basingstoke, and Hampshire, England). Fresh colonies were obtained by inoculating and incubating the suspension on MacConkey (Oxoid LTD, Basingstoke, Hampshire, England) and 5% sheep blood agar (HiMEDIA Laboratories Pvt. Ltd, Mumbai, India) for 18-24 hours at 37 ^{o}C. The pure colonies from the non selective agar were used for subsequent AST and ESBLs test.

5.11.3 Preparation of Clavulanate Stock Solution

For CDT method, the combined disks (Ceftazidime-clavulanate (30 µg/10 µg) and cefotaxime-clavulanate (30 µg/10 µg) disks) were prepared from in-house made clavulanate solution according to CLSI guideline (18). From potassium clavulanate analytical standard powder (Sigma-Aldrich Corp, St. Louis, MO USA) stock solution of clavulanate at 1000 µg/ml was prepared, aliquoted and stored at -70°C freezer. When we were ready to perform CDT (each day of testing), 10 µL of clavulanate solution was added to ceftazidime (30 µg) and cefotaxime (30 µg) disks (Abtek Biologicals Ltd, Liverpool, United Kingdom) and allowed to about 30 minutes for the clavulanate to absorb and the disks to be dry enough for application. The combined disks were used immediately (within 30 minutes) after they had dried.

5.11.4 Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing (AST) was carried out by the Kirby-Bauer disc diffusion method according to 26th edition CLSI guideline (18). After preparation of 0.5McFarland turbidity inoculums, Muller-Hinton (MHA) (Oxoid LTD, Basingstoke, Hampshire, England) plates was inoculated and antimicrobial discs was applied on the plate. The antibiotic discs used in this study were amoxicillin-clavulanic acid (AMC: 20/10 µg), cefotaxime (CTX: 30 µg), ceftazidime (CAZ: 30 µg), cefepime (FEP: 30 µg), Cefoxitin (30µg), meropenem (MER: 10 µg), gentamicin (GEN: 10 µg), amikacin (30µg) ciprofloxacin (CIP: 5 µg), norfloxacin (NOR: 10 µg) and sulfamethoxazole-trimethoprim (SXT: 3.75/1.25µg). The antibiotic discs were supplied Abtek Biologicals Ltd, Liverpool, United Kingdom. *Enterobacteriaceae* isolate was considered as MDR if it was non susceptible to three or more drugs of different classes of antibiotics (68).

5.11.5 Screening *Enterobacteriaceae* for ESBLs Production

Those *Enterobacteriaceae* which were resistant or reduced susceptibility to cefotaxime and/or ceftazidime (indicator cephalosporin(s)) were included as potential ESBL producers. In other word isolates that showed an inhibition zone size of ≤ 22 mm for ceftazidime (30 µg) and/or ≤27 mm for cefotaxime (30 µg) were considered as potential ESBL producers and selected for confirmation for ESBLs production using CDT as recommended by CLSI guidelines (18).

5.11.6 Confirmation of ESBLs with Combination Disc Test

A disc of ceftazidime (30 µg), cefotaxime (30 µg) and cefepime (30 µg), and ceftazidime + clavulanic acid (30 µg/10 µg), cefotaxime (30 µg) + clavulanic acid (30 µg/10 µg) and cefepime (30 µg) + clavulanic acid (30 µg/10 µg) was placed at appropriate distance on a MHA plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards and incubated overnight (18 – 24 hrs) at 37°C as per CLSI guideline. An increase in the inhibition zone diameter of ≥5 mm for a combination disc versus ceftazidime or cefotaxime disc alone was confirmed as ESBLs production (18). The Cefepime (30 µg) and cefepime (30 µg) + clavulanic acid (30 µg/10 µg) was used as per EUCAST's recommendation (69).

5.11.7 Confirmation of ESBLs with Double Disk Synergy Test

For the introduction of simple phenotypic methods for the detection and confirmation of ESBLs producing *Enterobacteriaceae*, the double disk synergy test (DDST) was tested and compared against CDT. The DDST was performed together with the AST according to the method developed by Jarlier and his collagues and recommended by EUCAST (20,69). Briefly, 0.5 McFarland suspension of the test isolate was swabbed on Muller Hinton Agar (MHA) plate and 30 µg antibiotic discs of ceftazidime, cefepime and cefotaxime were placed on the plate 20 mm (center to center) from the amoxicillin-clavulanate (20µg/10 µg) disc and incubated at 37 ^{0}C aerobically overnight. Clear extension of the edge of the inhibition zone of cephalosporin toward the amoxicillin-clavulanate disc was interpreted as positive for ESBLs production.

5.11.8 Quality Control

Standard Operating Procedures (SOP) were followed for every methods applied in this study. Expiry date of the media, reagents and antibiotic disks were checked before use. After sterility check, the culture media was inspected visually for cracks and thickness; and for the presence of freezing, bubbles, and contaminant. Quality control for new batch was performed using ATCC 25922 *E. coli* standard strain to check the quality of culture media and antibiotics disks. For ESBLs confirmatory test, *K. pneumoniae* ATCC® 700603 (ESBLs positive) and *E. coli* ATCC® 25922 (ESBLs negative) standard control strains was used (18).

5.12 Data Entry and Analysis

Data was entered and analyzed using SPSS Version 20 (IBM Corporation, Armonk, NY, USA). Proportions and percentage or frequency of ESBLs producing *Enterobacteriaceae* isolate was used to describe frequency outputs for categorical variables. Mean and Standard deviation was used to describe continuous variables. Sensitivity, specificity, positive and negative predictive values were calculated to compare DDST against CDT method. Binary logistic regression was used to see the relation of dependent and independent variables. The Chi-square test was used to compare the difference of resistance or sensitivity to antibiotics between the ESBLs-producing and the non-ESBLs-producing *Enterobacteriaceae*. $P \leq 0.05$ was considered statistically significant.

5.13 Data Quality Assurance

Before data entry, data from the data collection form was checked for its completeness and accuracy. Culture, antibiotics susceptibility test and ESBLs test results were recorded carefully before entry to SPSS. Furthermore, data cleaning and double-data check was applied in order to assure quality of the data.

5.14 Ethical Considerations

Ethical clearance for this study was obtained from the departmental research and ethics review committee (DRERC) of the medical laboratory sciences, school of allied health sciences, College of Health Sciences; Addis Ababa University. Permission was obtained from each selected laboratories.

5.15 Dissemination of Result

The result was submitted to the Department of Medical Laboratory Sciences, Addis Ababa University. The result will also be disseminated to the respective microbiology laboratories, Federal Ministry of Health and other concerned bodies related with this public health issue programs according to the university's and other ethical regulations. Finally the full manuscript will be published at International or national peer reviewed journal.

6 RESULTS

6.1 Demographic characteristics of the patients

A total of 426 consecutive *Enterobacteriaceae* isolates were collected from four microbiology laboratories. Of which 150 from International Clinical Laboratories (ICL), 118 from Tikur Anbessa Specialized Hospital (TASH), 89 from Ethiopian Public Health Institute (EPHI) Bacteriology Laboratory and 69 isolates from Yekatit 12 Medical College Hospital (YMCH). These isolates were identified from clinical specimens of different sources of infections. The majority, 272 (63.8%) of the *Enterobacteriaceae* were isolated from urine followed by blood 90 (21.1%). (**Table 6.1**)

Among all *Enterobacteriaceae*, 54.4% (236) of them were isolated from males. The most frequently isolate found among males were *E. coli* (47.4%) and *K. pneumoniae* (28.4%), similarly among female *E. coli* (58.5%) and *K. pneumoniae* (20.8%). The isolates were obtained from patients ranged from 1day to 91 years of age with the mean age of 32.6 years and standard deviation of 25.6. Among all *Enterobacteriaceae* isolates, 58/426 (13.6%) of them were isolated from infants less than 1 year, 93/426 (21.8%) from children less than 5 years and 135/426 (31.7%) from children less than 15 year of age (**Table 6.1**).

6.2 Frequency of *Enterobacteriaceae* Isolates

Among all *Enterobacteriaceae* the most frequent isolates were *E. coli with* 53.5 % (228/426) and *K. pneumoniae* 24.1 % (103/426). *E. coli* were predominantly isolated in urine with 82.5% (188/228) and 10.5% (24/228) from blood specimens. *K. pneumoniae* were obtained mostly from blood with 54.1% (53/103), followed by urine and wound/pus with 31.1% (32/103) and 11.6% (12/103) respectively (**Table 6.1**).

Table 6.1: Distribution of *Enterobacteriaceae* isolate against demographic characteristics, specimen types and bacteriology lab, at selected microbiology laboratories, Addis Ababa, Ethiopia from January 1 to May 30, 2017

Variables (Number)		***Enterobacteriaceae* isolate n (%)**						
		E .coli	***K.pneumoniae***	***E. cloacae***	***Citrobacter . Spps***	***K.oxytoca***	***K.ozanae***	**Other isolate**
Gender	Male (190)	90 (47.4)	54 (28.4)	17 (8.9)	17 (8.9)	5 (2.6)	1 (0.5)	6 (3.1)
	Female (236)	138 (58.5)	49 (20.8)	5 (2.1%)	12 (5.0)	10 (4.2)	12 (5.1)	10 (4.2)
Age group	≤ 28days (24)	3 (12.5)	**18 (75.0)**	0 (0.0)	0 (0.0)	0 (0.0)	3 (12.5)	0 (0.0)
	29days-<1year (34)	9 (26.5)	**21 (61.8)**	0 (0.0)	1 (2.9)	2 (5.9)	0 (0.0)	1 (2.9)
	1- <5 years (35)	16 (45.7)	9 (25.7)	1 (2.9)	1 (2.9)	1 (2.9)	0 (0.0)	7 (20.0)
	5- <15 years (42)	18 (42.9)	14 (33.3)	2 (4.8)	1 (2.9)	3 (7.1)	0 (0.0)	4 (9.5)
	15-<25 years (35)	14 (40.0)	5 (14.3)	4 (11.4)	5 (14.3)	4 (11.4)	3 (8.6)	0 (0.0)
	25-<65 years (190)	**119 (62.6)**	30 (15.8)	7 (3.7)	18 (9.5)	4 (2.1)	5 (2.6)	7 (3.7)
	≥ 65 years (66)	**49 (74.2)**	6 (9.1)	4 (6.1)	4 (6.0)	1 (1.5)	2 (3.0)	0 (0.0)
Bacteriology laboratories	ICL (150)	**109 (72.7)**	9 (6.0)	6 (4.0)	13 (8.7)	3 (2.0)	6 (4.0)	4 (2.6)
	EPHI (89)	36 (40.4)	29 (32.6)	6 (6.7)	6 (6.7)	2 (2.2)	4 (4.5)	6 (6.7)
	TASH (118)	53 (44.9)	33 (28.0)	7 (5.9)	9 (7.6)	9 (7.6)	1 (0.8)	6 (5.1)
	YHMC (69)	30 (43.5)	32 (46.4)	3 (4.3)	1 (1.4)	1 (1.4)	2 (2.9)	0 (0.0)
Types of Specimen	Urine (272)	188(69.1)	32 (11.8)	11 (4.0)	19 (7.0)	7 (2.6)	7 (2.6)	8 (2.9)
	Blood (90)	24 (26.7)	53 (58.9)	3 (3.3)	2 (2.2)	5 (5.6)	3 (3.3)	0 (0.0)
	Pus (40)	8 (20.0)	12 (30)	6 (15.0)	4 (10.0)	2 (5.0)	3 (7.5)	5(12.5)
	Sputum (6)	1(16.7)	2 (33.3)	1 (16.7)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
	CSF (2)	0(0.0)	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Body fluids (11)	5(45.5)	2 (18.2)	1 (9.1)	1 (9.1)	1 (9.1)	0 (0.0)	0 (0.0)
	Ear & Eye discharge(5)	2(40.0)	0 (0.0)	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	2(40.0)
	Total (N=426)	228(53.5)	103(24.1)	22 (5.2)	29 (6.8)	15 (3.5)	13 (3.1)	16(3.8)

Note: *Other isolates are *P.mirabilis*, *Providencia spps, M. morgani* and *E. aerogens*

6.3 Antibiotics Resistance pattern of *Enterobacteriaceae*

The antibiotics resistance pattern of *Enterobacteriaceae* isolated in different clinical specimens against 11 antibiotics is presented in **Table 6.2.** Highest resistance level was recorded to sulfamethoxazole-trimethoprim (77.0%), followed by amoxicillin with clavulinic acid (71.6%), cefotaxime (62.2%), cefepime (60.3%), ceftazidime (60.8%) and norfloxacillin (58.8%). There were also significant level of resistance to ciprofloxacin (46.3%), gentamycin (43.4%) and cefoxitin (25.1%). Lower resistance levels were observed against meropenem (5.2%) and amikacin (13.8%).

Escherichia coli showed highest resistance to sulfamethoxazole-trimethoprim (77.6%) followed by amoxicillin-clavulinic acid (70.0%), norfloxacin (64.3%) and ciprofloxacin (64.0%). In addition, its resistance level to cefotaxime, cefepime and ceftazidime was 54.8%, 53.5%, and 53.1% respectively. However, lowest level of resistance was observed to MER (3.5%) and AMK (11.8%). In *K. pneumoniae* the highest level of resistance was observed against cefotaxime (86.4%), cefepime (85.4%), ceftazidime (85.4%), amoxicillin-clavulinic acid (85.4%) and gentamicin (70.0%); while low resistance level to meropenem (10.7%) and amikacin (21.3%). **(Table 6.2)**

The overall multi-drug resistance MDR (non-susceptible to at least 3 antibiotics belonging to different antibiotics categories) level of *Enterobacteriaceae* isolate was 68.3% (291/426). From the 68.3% MDR *Enterobacteriaceae*, *E. coli* was the predominant species *with* 150 (35.0%) followed by *K. pneumoniae* 85 (20.0%). The highest MDR level was recorded in *K. pneumoniae* having 83.5% (86/103) followed by *Citrobacter species, E. coli, and E. cloacae* 68.9% (20/29), 66.2% (151/228) and 63.6% (14/22) respectively. None of *P. mirabilis* was found to be MDR. Only 11.3% (48/426) *Enterobacteriaceae* were susceptible for all tested antibiotics (**Table 6.3**). From the total MDR *Enterobacteriaceae* the predominant were *E. coli* 51.9% (151/291) and K. *pneumoniae* 29.6% (86/921) **(Figure 6.1).**

Table 6.2: Antimicrobial resistance pattern of *Enterobacteriaceae* isolated from different clinical specimens at selected microbiology laboratories in Addis Ababa, Ethiopia from January 1 to May 30, 2017.

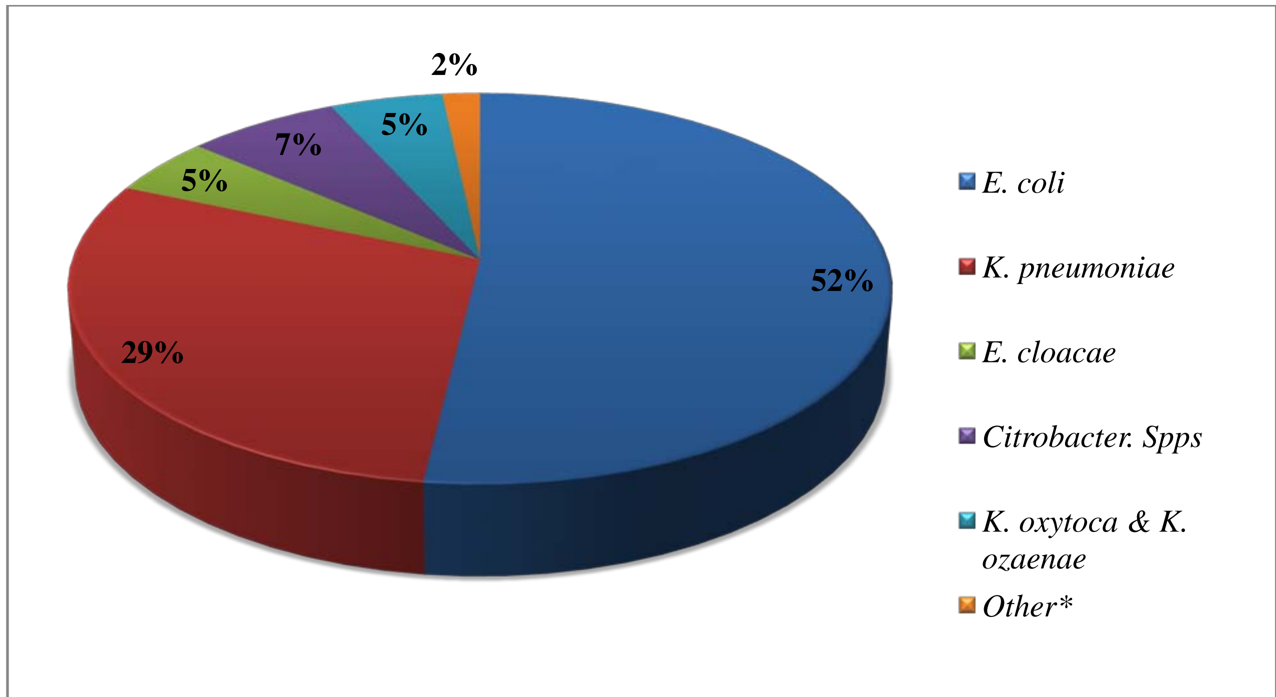
Isolates (number)	Tested Antibiotics Number (n (%))										
	CTX	**CAZ**	**CFP**	**FOX**	**MER**	**SXT**	**CPR**	**GEN**	**AMK**	**AMC**	**NOR N/Total**
E. coli **(n=228)**	125 (54.8)	121 (53.1)	122 (53.5)	50 (21.9)	8 (3.5)	177 (77.6)	146 (64.0)	76 (33.3)	27 (11.8)	160 (70.0)	121/188 (64.3)
K. pneumoniae **(n=103)**	89 (86.4)	88 (85.4)	88 (85.4)	24 (23.3)	11 (10.7)	89 (86.4)	52 (50.5)	72 (70.0)	22 (21.3)	87 (85.4)	18/32 (56.2)
E. cloacae **(n=22)**	12 (54.5)	11 (50.0)	12 (54.5)	14 (63.6)	0 (0.0)	14 (63.6)	9 (40.9)	9 (40.9)	0 (0.0)	16 (72.7)	3/11 (27.3)
C. diversus **(n=19)**	12 (63.2)	12 (63.2)	12 (63.2)	5 (26.3)	1 (5.3)	15 (78.9)	12 (63.2)	10 (52.6)	0 (0.0)	13 (68.4)	6/6 (100)
K. oxytoca **(n=15)**	8 (53.3)	8(53.3)	7 (46.7)	2 (13.3)	1 (6.7)	9 (60.0)	5 (33.3)	5 (33.3)	1 (6.7)	8 (53.3)	3/7(42.8)
K. ozaenae **(n=13)**	7 (53.8)	7 53.8)	6 (46.2)	2 (15.4)	0 (0.0)	8 (61.5)	6 (46.2)	5 (38.5)	0 (0.0)	8 (61.5)	0/5 (0.0)
Citrobacter. Spps **(n=10)**	5 (50.0)	5(50.0)	5 (50.0)	7 (70.0)	1 (10)	7 (70.0)	5 (50.0)	3 (30.0)	0 (0.0)	5 (50.0)	5/7 (71.4)
Providencia spps. **(n=7)**	3 (42.8)	3(42.8)	3 (42.8)	2 (28.6)	0 (0.0)	3 (42.8)	2 (28.6)	1 (14.3)	0 (0.0)	4 (57.1)	2/4 (50.0)
P. mirabilis **(n=5)**	1(20)	1(20)	1(20)	0(0.0)	0(0.0)	3(60)	2(40)	1(20)	0(0.0)	0(0.0)	0/1 (0)
M. morganii **(n=2)**	2(100)	2(100)	2(100)	1(50)	0(0)	2(100)	1(50)	1(50)	0(0)	2(100)	0(0)
E. aerogens **(n=2)**	1(50)	1(50)	1(50)	0(0)	0(0)	1(50)	0(50)	1(50)	0(0)	1(50)	0/2(0)
Total Resistance (N=426)	**265 (62.2)**	**257 (60.8)**	**259 (60.3)**	**107 (25.1)**	**22 (5.2)**	**324 (77.0)**	**240 (46.3)**	**185 (43.4)**	**59 (13.8)**	**305 (71.6)**	**160/272 (58.8)**

Note: CTX-Cefotaxime, CAZ-Ceftazidime, FOX-Cefoxitin CFP-Cefepime, MER-Meropenem CPR-Ciprofloxacin, NOR-Norfloxacin SXT-Sulfamethoxazole-trimethoprim, GEN-Gentamycin, AMK-Amikacin, AMC-Amoxicillin-Calvulanic acid.

Table 6.3: Multidrug resistance level of *Enterobacteriaceae* isolated different clinical specimen at the four microbiology laboratories, Addis Ababa, Ethiopia from January1 to May 30, 2017.

Isolates (number)	Level of antibiotics resistance ((number (%))								Total MDR-E (≥R3)
	RO	R1	R2	R3	R4	R5	R6	R7	
***E. coli* (228)**	20 (8.8)	28(12.3)	29(12.7)	34(14.9)	44(19.3)	52(22.8)	16(7.0)	5(2.2)	**151(66.2)**
***K. pneumoniae* (103)**	6(5.8)	5(4.8)	7(6.8)	10(9.7)	22(21.3)	33(32.0)	11(10.7)	9(8.7)	**86(83.5)**
***E. cloacae* (22)**	4(18.2)	2(9.1)	2(9.1)	3(13.6)	2(9.1)	3(13.6)	6(27.3)	0(0.0)	**14(63.6)**
***C. diversus* (19)**	3(15.8)	2(10.5)	1(5.3)	1(5.3)	3(15.8)	5(26.3)	3(15.8)	1(5.3)	**13(68.4)**
***K. oxytoca* (15)**	5(33.3)	2(13.3)	1(6.7)	0(0.0)	2(13.3)	4(26.7)	0(0.0)	1(6.7)	**7(46.6)**
***K. ozaenae* (13)**	1(7.7)	3(23.1)	1(7.7)	2(15.4)	5(38.5)	1(7.7)	0(0.0)	0(0.0)	**8(61.5)**
***Citrobacter. Spps* (10)**	3(30.0)	0(0.0)	0(0.0)	3(30.0)	0(0.0)	2(20.0)	1(10.0)	1(10.0)	**7(70)**
***Providencia Spps*.(7)**	3(42.8)	0(0.0)	1(24.3)	0(0.0)	2(28.5)	1(24.3)	0(0.0)	0(0.0)	**3(42.8)**
***P. mirabilis* (5)**	2(40.0)	1(20.0)	2(20.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	**0(0.0)**
***M. morganii* (2)**	0(0.0)	0(0.0)	0(0.0)	1(50.0)	0(0.0)	0(0.0)	1(50.0)	0(0.0)	**1(50.0)**
***E. aerogens* (2)**	1(50.0)	0(0.0)	0(0.0)	0(0.0)	1(50.0)	0(0.0)	0(0.0)	0(0.0)	**1(50.0)**
Total(N=426)	**48(11.3)**	**43(10.1)**	**43(10.1)**	**54(12.7)**	**81(19.0)**	**101(23.7)**	**38(8.9)**	**17(4.0)**	**291(68.3)**

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.



Other=Providencia Spps*.(3), *M. morganii (1) E. aerogens (1)*

Figure 6.1: Distribution of major MDR isolate among the total MDR *Enterobacteriaceae* in selected microbiology, Addis Ababa, Ethiopia, January 1to May 30, 2017

6.4 Magnitude of ESBLs Producing *Enterobacteriaceae*

Of all *Enterobacteriaceae* 62.2% (265/426) were found to be suspected ESBLs producing *Enterobacteriaceae* with cefotaxime zone of inhibition ≤ 27 mm and ceftazidime zone of inhibition ≤ 22 mm.

Of the 265 ESBLs suspicious *Enterobacteriaceae*, ESBLs production was confirmed in 246 (92.8%) of isolates by the combination disk test**.** The overall magnitude of ESBLs producing *Enterobacteriaceae* was 57.7% (246/426) which constitute *E. coli* 27.9% (119/426), *K. pneumoniae* 19.0% (81/426) and other *Enterobacteriaceae* 10.8% (46/426).

The distribution of ESBLs producers varied among different species of *Enterobacteriaceae*. The highest intra-species frequency of ESBLs production was observed among *K. pneumoniae* 78.6% (81/103) followed by *E. coli and Citrobacter species* with 52.2% (119/228) *and* 51.7% (15/29) respectively **(Figure 6.3).** The lowest intra-species ESBLs production was observed in *P. mirabilis with* 20% (1/5) proportion.

Regarding ESBLs producing *Enterobacteriaceae* distribution across age groups, the highest proportion was observed among isolates from patients less than 1 year (86.2%), ≤ 28days (87.5%) and 5 to <15 years (69.0%) than other age groups. The total proportion of ESBLs producing *Enterobacteriaceae* among less than 15 years children was 74.1% (100/135) (**Table 6.1**). The occurrence of ESBLs was statistically significant among *Enterobacteriaceae* isolated from age group 29days to <1year (85.3%) ($P < 0.05$).

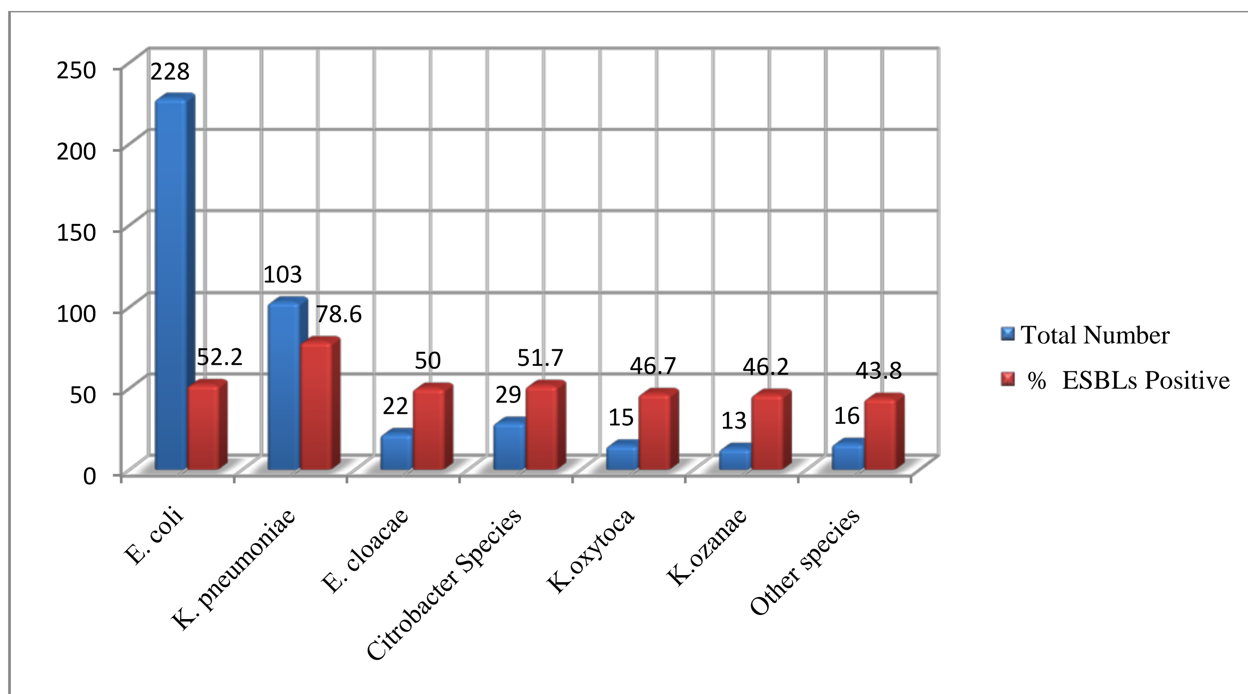


Figure 6.3: Frequency of ESBLs producing *Enterobacteriaceae* species from different clinical specimens at selected microbiology laboratory in Addis Ababa, Ethiopia from January 1 to May 30, 2017

6.5 Distribution of MDR and ESBLs producing *Enterobacteriaceae*

The magnitude of ESBLs producing *Enterobacteriaceae* (ESBLs-E) was different in the four microbiology laboratory. The magnitude of ESBLs-E was highest in TASH 71.5% (84/118) followed by YHMC 68.1% (47/69), EPHI clinical bacteriology laboratory 66.3% (59/89) and lowest in ICL 37.3% (56/150). In all laboratories highest ESBLs production was observed among *K. pneumoniae* 78.6% (81/103). Distribution of MDR- *Enterobacteriaceae* and major ESBLs producing *Enterobacteriaceae* at the four microbiology laboratories is presented in **Table 6.4**.

Table 6.4: Distribution of major ESBLs producing *Enterobacteriaceae* with their MDR level at the four microbiology laboratories, Addis Ababa, Ethiopia from January 1 to May 30, 2017

Isolate collection Site	ESBLs producing *Enterobacteriaceae* (n (%))	Major ESBLs producing *Enterobacteriaceae*			MDR *Enterobacteriaceae* (n (%))
		E. coli (n (%))	*K. pneumoniae* (n (%))	*E. coli* and *K. pneumoniae* (n (%))	
TASH	**71.5** (84/118)	**64.2** (34/53)	**84.8** (28/33)	**72.1** (62/86)	**79.7** (94/118)
YHMC	**68.1** (47/69)	53.3 (16/30)	**84.4** (27/32)	**69.3** (43/62)	**71.0** (49/69)
EPHI	**66.3** (59/89)	**75.0** (27/36)	**72.4** (21/29)	**73.8** (48/65)	**70.8** (63/89)
ICL	37.3 (56/150)	38.5 (42/109)	55.6 (5/9)	39.8% (47/118)	56.7 (85/150)
Total	57.7(246/426)	52.2 (119/228)	**78.6** (81/103)	60.4 (200/331)	68.3 (291/426)

6.6 Distribution of ESBLs producing *Enterobacteriaceae* with their MDR level among different specimens

From all specimen included in this study, the highest magnitude of ESBLs-E 84.4% (76/90) and MDR 83.3% (75/90) was found in blood. In urine specimen, the extent of ESBLs-E and MDR were 50.7% (138/272) and 66.5% (181/272) respectively (**Table 6.5**). Of all ESBLs producing *Enterobacteriaceae*, 96.3% (237/246) were MDR, whereas only 30% (54/180) of the non–ESBLs producers were MDR. Being ESBLs producer has statistically significant association with MDR ($P < 0.001$). That is, the odd of being MDR were 61.4 times (95% CI COR=29.37-128.53) more likely among ESBLs producing *Enterobacteriaceae* than non ESBLs isolate.

Table 6.5: Distribution of ESBLs producing *Enterobacteriaceae* with their MDR level in different clinical specimens at the selected microbiology laboratories, Addis Ababa, Ethiopia January 1 to May 30, 2017

Specimens (number)	MDR-E n (%)		ESBLs test result n (%)		
	YES	NO	POS	NEG	Non-ESBL Suspicious
Urine (272)	181 **(66.5)**	91 (33.5)	138 (50.7)	14 (5.1)	120 (44.1)
Blood (90)	75 **(83.3)**	15 (16.7)	76 **(84.4)**	3 (3.3)	11 (12.2)
Wound or Pus (40)	23 (57.5)	17 (42.5)	21 (52.5)	2 (5.0)	17 (42.5)
Other specimens* (24)	12 (50.0)	12 (50.0)	11 (45.8)	0 (0.0)	13 (44.2)
Total (N=426)	291 (68.3)	135 (31.7)	246 (57.7)	19 (4.5)	161 (37.8)

Note: * Other specimens: CSF & other body fluids, sputum, ear and eye discharge

6.7 Association of independent variables with magnitude of ESBLs-E

In multivariate analysis using logistic regression, magnitude of ESBLs-E has statistically significant association with age group, microbiology laboratory and type of specimens.

Enterobacteriaceae isolate that are isolated from age group 29days - <1year are 3.933 (95% AOR = 1.149-13.456, p = 0.029) times more likely to be ESBLs-E than other age group. The chances of getting ESBLs positive among *Enterobacteriaceae* in TASH microbiology laboratory is 3.08 (95% CI = 1.69 - 5.61, p < 0.001) times higher than *Enterobacteriaceae* in ICL. However, there was no statistical significance between male and female for acquisition of ESBLs-E **(Table 6.6)**.

Table 6.6: The association of independent variables with magnitude of ESBLs, at selected microbiology laboratories, Addis Ababa, Ethiopia from January 1 to May 30, 2017

Variables (Number)	*ESBL-Pos* n (%)	*COR (95% COR)*	*P value*	*AOR(95% AOR)*	*P value*
Gender					
Male (190)	110 (57.9)	1.011(0.687-1.488)	0.0956		
Female (236)	136 (57.6)	Ref*			
Age group					
≤ 28days (24)	21 (87.5)	8.312 (2.090-33.057)	0.003	3.763 (0.822-17.221)	0.088
29days-<1year (34)	29 (85.3)	6.888 (2.162-21.946)	0.001	3.933 (1.149-13.456)	0.029**
1- <5 years (35)	21 (60.0)	1.781(0.690-4.599)	0.233	1.29 (0.46-3.63)	0.622
5- <15 years (42)	29 (69.0)	2.469 (1.042-6.733)	0.041	1.88 (0.69-5.07)	0.214
15-<25 years (35)	16 (45.7)	Ref*			
25-<35 years (68)	34 (50.0)	1.188 (0.524-2.689)	0.680	1.65 (0.68-3.99)	0.262
35-<45 years (43)	21 (48.8)	1.134 (0.463-2.772)	0.784	1.58 (0.61-4.09)	0.350
45-<55 years (41)	20 (48.8)	1.131 (0.458-2.793)	0.790	1.36 (0.52-3.58)	0.526
55-<65 years (38)	22 (57.9)	1.633 (0.647-4.121)	0.299	2.11 (0.78-5.72)	0.140
≥ 65 years (66)	33 (50.0)	1.188 (0.522-2.701)	0.682	1.65 (0.68-4.00)	0.272
Microbiology laboratories					
TASH (118)	84 (71.2)	4.147 (2.171-6.961)	0.000	3.080 (1.692-5.608)	0.000**
YHMC (69)	47 (68.1)	3.586 (1.959-6.566)	0.000	2.239 (1.153-4.346)	0.017**
EPHI (89)	59 (66.3)	3.301 (1.904-5.722)	0.000	2.796 (1.508-5.184)	0.001**
ICL (150)	56 (37.3)	Ref*			Ref*
Types of Specimen					
Blood (90)	76 (84.4)	5.43 (2.560-11.51)	0.000	3.223(1.379-7.532)	0.007**
Urine (272)	138(50.7)	1.03 (0.597-1.775)	0.916	1.360 (0.746-2.476)	0.315
Pus and other specimens*** (40+24=64)	32 (50.0)	Ref*			Ref*

Note: ** Reference*

*** There is statistical significant association between the variables and ESBLs magnitude*

****Other specimens are CSF (2), body fluids (11), sputum (6), and eye and year discharge (5)*

6.8 Association of ESBLs status of *Enterobacteriaceae* with antibiotics resistance level

In general, *Enterobacteriaceae* that produce EBSLs were more resistant to the antibiotics than non–ESBLs producers. Almost all of ESBLs positive and ESBLs negative *Enterobacteriaceae* were resistance to the 3rd and 4th generation cephalosporin. However, it was only 8 (3.3%) of ESBLs positive isolate that were resistance to meropenem. The possibility of ESBLs positive *Enterobacteriaceae* to become resistance to other antibiotics was statistically significant (P value < 0.05) among ESBLs producing isolate than non ESBLs suspicious isolate. For example, the odd of *Enterobacteriaceae* to become resistant to cefoxitin is 4.52 (P = 0.000) times more likely among ESBLs producers than non-ESBLs suspicious. Furthermore, ESBLs negative (i.e ESBLs suspicious isolates) were highly resistant than non-ESBLs suspicious isolate (P value < 0.05). For example, the probability of *Enterobacteriaceae* to become resistance to amikacin is 23.3 (P = 0.000) times more likely among ESBLs negative than non-ESBLs suspicious isolates. **(Table 6.7)**

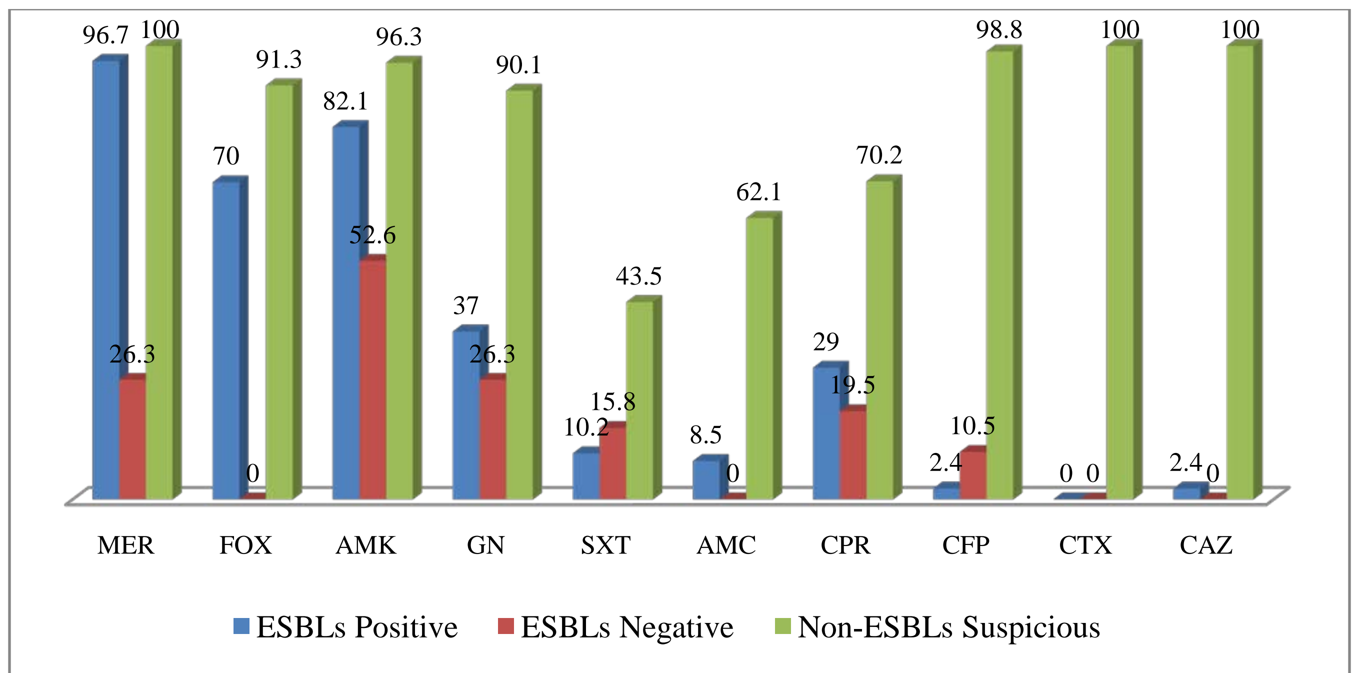
Table 6.7: Association of ESBLs status of *Enterobacteriaceae* with their antibiotics resistance level, at selected microbiology laboratories, Addis Ababa, Ethiopia from January 1 to May 30, 2017

ESBLs Status (Independent variable)	Resistance level of *Enterobacteriaceae* to the Antibiotics (Dependent variable)															
	CTX	CAZ	CFP	MER	FOX		AMC		AMK		GEN		SXT		CPR	
	n (%)	n (%)	n (%)	n (%)	n (%)	P value (OR)	n (%)	P value (OR)	n (%)	P value (OR)	n (%)	P value (OR)	n (%)	P value (OR)	n (%)	P value (OR)
ESBLs POS (n=246)	246 (100)	240 (97.6)	240 (97.6)	8 (3.3)	74 (30.1)	0.000 (4.52)	225 (91.5)	0.000 (17.5)	44 (17.9)	0.000 (5.63)	155 (63.0)	0.000 (15.4)	221 (89.8)	0.000 (6.8)	176 (71.5)	0.000 (5.92)
ESBLs NEG (n=19)	19 (100)	19 (100)	17 (89.5)	14 (73.7)	19 (100)	NA	19 (100)	NA	9 (47.4)	0.000 (23.3)	14 (73.7)	0.000 (25.4)	16 (84.2)	0.030 (4.1)	16 (84.2)	0.000 (12.6)
NON-ESBLs Suspicious (n=161)	0 (0.0)	0 (0.0)	2 (1.2)	0 (0.0)	14 (8.7)	Ref*	61 (37.9)	Ref*	6 (3.7)	Ref*	16 (9.9)	Ref*	91 (56.5)	Ref*	48 (29.8)	Ref*

Ref*=Reference. OR= Odd ratio, NA= Not applicable i.e OR is not calculated one of the 2x2 table box is zero. For CTX, CAZ, CFP and MER the SPSS do not calculate OR because one of the 2x2 table box is zero.

6.9 Antibiotics susceptibility of ESBLs producing *Enterobacteriaceae* to potentially active drugs

In general, non-EBSLs producing *Enterobacteriaceae* were more sensitive to the antibiotics than ESBLs producers (P value < 0.05). The most active drugs for ESBLs producing *Enterobacteriaceae* were meropenem, amikacin and cefoxitin with sensitivity of 96.7%, 82.1% and 70% respectively. Moreover, 37%, 29% and 10.2% of the ESBLs producing isolates were sensitive to gentamicin, ciprofloxacin and cotrimoxazole respectively. In other side, ESBLs negative *Enterobacteriaceae* have low sensitive to the antibiotics, for instance 52.6% to amikacin, 26.3% to meropenem and 26.3% gentamicin. Non-ESBLs suspicious *Enterobacteriaceae* were 100% sensitive to meropenem, cefotaxime and ceftazidime. Furthermore, 96.3%, 91.3% and 90.1% of non-ESBLs suspicious isolates were sensitive to amikacin, cefoxitin and gentamicin respectively. (**Figure 6.3**)



Note: MER: meropenem FOX: Cefoxitin, AMK: amikacin, GN: gentamicin, SXT: trimethoprim–sulfamethoxazole, AMC: amoxicillin–clavulinic acid, CPR: ciprofloxacin, CEF:Cefepime,

Figure 6.3: Antibiotics susceptibility pattern of ESBLs positive, ESBLs negative and Non-ESBLs Suspicious *Enterobacteriaceae* to different classes of antibiotics at selected microbiology laboratories, Addis Ababa, Ethiopia January 1 to May 30 2017

6.10 Comparison of double-disk synergy method against combination disk method

For the purpose of introduction of simple, cheap and applicable ESBLs confirmatory method to bacteriology laboratories, DDST was compared against CDT. All 426 *Enterobacteriaceae* isolates were screened for ESBLs production using cefotaxime and ceftazidime antibiotics disks. Hence, cefotaxime identified 265 and ceftazidime 259 as potential ESBLs producing isolates. Of 265 Enterobacteriaceae which were suspicious for ESBLs, 92.8% (246/265) were confirmed as ESBLs producer by CDT. The DDST, which was performed in conjunction with a routine disk diffusion test, detected ESBLs production in 224 out of 265 (84.5%) of the suspicious isolates (**Figure 6.4a and 6.4b**).

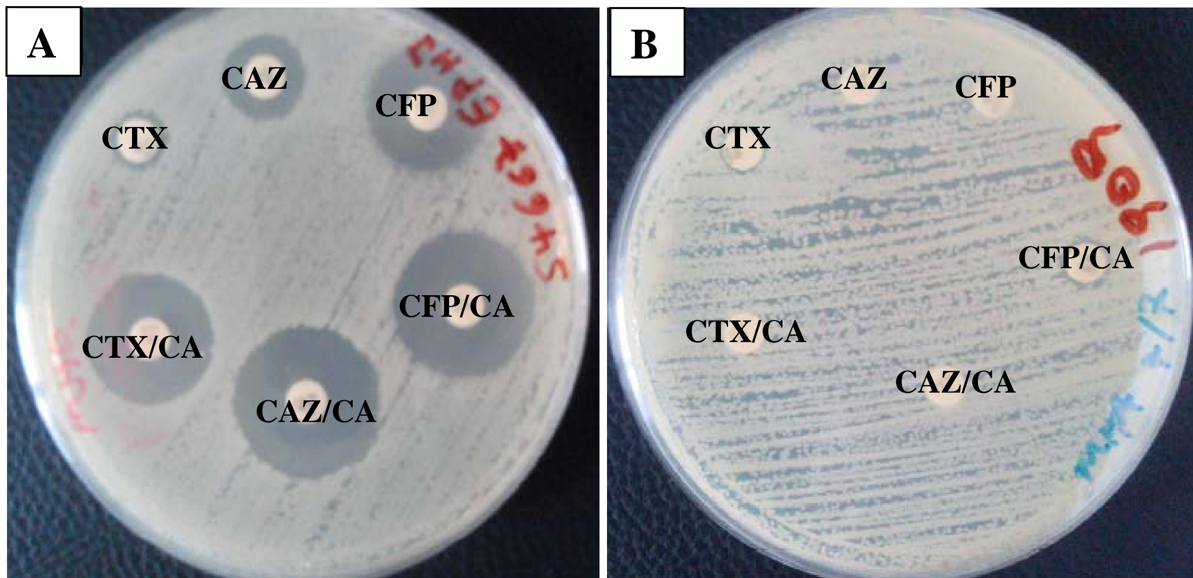


Figure 6.4a: Combination Disk Test on 90mm plate: ESBLs positive (A) and negative (B) *Enterobacteriaceae* isolates from different clinical specimens at selected microbiology laboratories in Addis Ababa, Ethiopia from January 1 to May 30, 2017

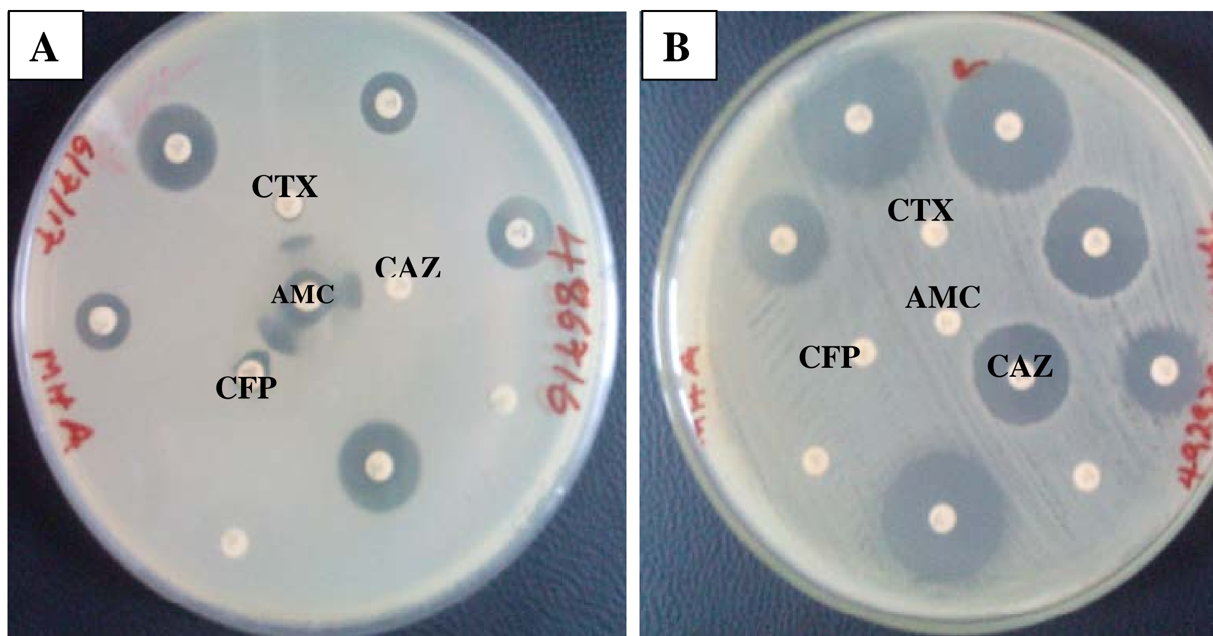


Figure 6.4b: Double Disk Synergy Test on 150mm plate: ESBLs positive (A) and negative (B) *Enterobacteriaceae* isolates from different clinical specimens at selected microbiology laboratories in Addis Ababa, Ethiopia from January 1 to May 30, 2017.

Of 246 ESBLs positive *Enterobacteriaceae* by CDT, Double Disk Synergy Test (DDST) identified 224 (91.1%) of them by making synergy with one or/and two or three cephalosporins. Of 224 DDST ESBLs positive isolate, 13 (5.8%) isolate made synergy only with cefepime. The rest 207 isolates with the three disks and 4 isolates with cefotaxime and cefepime. The measure of agreement or kappa value between CDT and DDST was 0.896.

When DDST was compared to CDT (which was taken as a reference method), it had a sensitivity of 91.1% (224/246), a specificity of 100% (19/19) and a positive predictive value of 100% (224/224) and negative predictive value of 46.3% (19/41).

7 DISCUSSIONS

ESBLs producing *Enterobacteriaceae* have become worldwide serious problem. Dissemination of ESBLs compromises the activity of broad-spectrum antibiotics creating major therapeutic difficulties with a significant impact on the outcome of patients. The continued emergence of ESBLs presents diagnostic challenges to the clinical microbiology laboratories (70).

7.1 Frequency of *Enterobacteriaceae* isolates

From all *Enterobacteriaceae* isolates the majority were *E. coli* 53.5% (228/426) followed by *K. pneumoniae* 24.1% (103/426). Previous studies in different part of Ethiopia have also reported comparable figure to our finding; *E. coli* and *K. pneumoniae* as the most common *Enterobacteriaceae* which were prevalent in clinical specimens, in Bahirdar: *E. coli* 58.1% *and K. pneumoniae* 23.3% (64), in Jimma: *E. coli* 75.9% *and K. pneumoniae* 24.1% (65). Studies from other countries also reported similar finding; in India *E. coli* (65.82%) and *K. pneumoniae* (24.9%) (52) ,in Burkina Faso *E. coli* (65.6%) and *K. pneumoniae* (22.7%) (71), and in Uganda *E. coli* (53.9%) and *K. pneumoniae* (28.9%) (63). Like other studies, our finding indicated that many types of infectious disease caused by *Enterobacteriaceae* were predominantly by *E. coli and K. pneumoniae*.

7.2 Antibiotics Resistance pattern of *Enterobacteriaceae* isolates

In the present study, high resistance was observed to sulfamethoxazole-trimethoprim (77.0%) followed by amoxicillin with clavulinic acid (71.6%), cefotaxime (62.2%), cefepime (60.3), ceftazidime (60.8%), norfloxacillin (58.8%), ciprofloxacin (46.3%) and gentamycin (43.4%). The results of this study was in line with the findings of studies conducted in Iran: sulfamethoxazole-trimethoprim (94%), gentamycin (57.8%), ceftazidime (73%) and ciprofloxacin (55.5%) (72); in Nepal: sulfamethoxazole-trimethoprim (62.1%), ceftazidime (83.2%), cefotaxime (74.7%), ciprofloxacin (61.1%) and norfloxacillin (64.2) (73) and in Sierra Leone; ceftazidime (62.9%), ciprofloxacin (74.2%) and gentamycin (74.3%) (74).

The resistance level of *Escherichia coli* to sulfamethoxazole-trimethoprim (77.6%), amoxicillin-clavulinic acid (70.0%), norfloxacin (64.3%) and ciprofloxacin (64.0%) in our study was concordant with study done in Dessie sulfamethoxazole-trimethoprim (65.1%) (75), in Gondar sulfamethoxazole-trimethoprim (78.3%) (76), in Tanzania: sulfamethoxazole-trimethoprim (76%) (77) and in Khartoum-Sudan: sulfamethoxazole-trimethoprim (88.3%), amoxicillin-clavulinic acid (51.4%) and CPR (58.4%) (78). However, it was lower than a study conducted in Iran: sulfamethoxazole-trimethoprim (92.8%) (72) and in Equatorial Guinea: sulfamethoxazole-trimethoprim (95%), amoxicillin-clavulinic acid (88.4%) and ciprofloxacin (59.8%) (79).

In *K. pneumoniae* the highest level of resistance was observed against sulfamethoxazole-trimethoprim (86.4%) cefotaxime (86.4%), cefepime (85.4%), ceftazidime (85.4%), amoxicillin-clavulinic acid (85.4%), gentamicin (70.0%) and ciprofloxacin (50.5%). There were also similar findings from studies conducted in Iran: sulfamethoxazole-trimethoprim (91.4%), ceftazidime (91.4%) and gentamicin (82.8%) (72); in Sierra Leone: ciprofloxacin (73.4%) and gentamicin (60%) (74), in Equatorial Guinea: sulfamethoxazole-trimethoprim (100%), amoxicillin-clavulinic acid (96.6%), gentamicin (86.2%) and ciprofloxacin (87.5%) (79). The high resistance of *Enterobacteriaceae* to the antibiotics may be due to the misuse, overuse of the antibiotics coupled with weak infection control measures (70).

In the present study, the overall magnitude of MDR among all *Enterobacteriaceae* isolate was 68.3%, which was fairly similar with a study done in Dessie (74.6%) (75), Gondar (68%) (80) and Nepal (64.04%) (81). However, our result was lower than studies done in different parts of Ethiopia such as in Gondar (93.5% and 87.4%) (76,82), in Bahirdar (93.1%) (83), in Nepal (96.84%) (73) and in Sierra Leone (85.7%) (74). The difference in magnitude of MDR isolates might be due to selection of antibiotic from different class, definition for MDR, study period and specimen type, and difference in study population.

There was intra-species difference in MDR level. The present study showed that the level of MDR in *K. pneumoniae* (83.5%) and *E. coli* (66.2%) was comparable with a study conducted in Equatorial Guinea in *E. coli* (74.4%) (79), in Sierra Leone *K. pneumoniae* (73.3%) and *E. coli* (61.5%) (74), However, our result is lower than a study conducted in Gondar, where the level in *K. pneumoniae* was (95.6%) and in *E. coli* was (92.9%) (82), in Khartoum-Sudan *E. coli* (92.2%)

(78), and in Equatorial Guinea in *K. pneumoniae* (91.7%) (79). The MDR level among *E. coli* (50.2%) in Dessie is lower than our study (75). The difference in MDR level among *K. pneumoniae and E. coli* in our study might be due to most *K. pneumoniae* was isolated from blood specimens that were collected from inpatients in the hospitals.

7.3 Magnitude of Extended Spectrum Beta-lactamase producing *Enterobacteriaceae*

Although no nationwide study has been conducted so far for detection of ESBLs producing *Enterobacteriaceae* in Ethiopia, few studies have been done in some parts of the country (65,66,84,85). In the present study magnitude of ESBLs producing *Enterobacteriaceae* was 57.7%, which was higher than studies reported by previous researchers in Ethiopia: 38.4%, 36%, 33.3% and 25% in Jimma by Siraj and his colleagues (65), in Jimma by Mulualem Y and his colleagues (84), Harar (85) and Adama (66) respectively. The present finding is worrisome, because 57.7% ESBLs producing *Enterobacteriaceae* isolates is higher than those studies done previously in different part of Ethiopia. The emergence of ESBLs producing *Enterobacteriaceae* in high magnitude emphasizes the need to implement strong infection control strategies to reduce ESBLs burden.

In the present study, magnitude of ESBLs producing *Enterobacteriaceae* (57.7%) was in agreement with a study done in Gahna 49.3% (56), Uganda 62.0% (63), Bahir-Dar 57.6% (64), Burkina Faso 58.0% (71), and Karnataka, India 57.5% (87). It has been indicated that one of the most important factors in the emergence of ESBLs production is the selective pressure caused by the use of 3rd generation cephalosporins (88,89). Lack of antibiotic surveillance, antibiotics misuse, and limited resources for sanitation and infection control strategies may have also contributed for the high magnitude of ESBLs.

Compared to our study, ESBLs producing *Enterobacteriaceae* prevalence in Europe vary between 0.7% in Austria and 23.8% in Turkey (90), and in Cairo-Egypt (16%) (91), Nepal (24.4%) (92), Italy (6.3%) (93), which were lower our study. This difference might be due to good infection control strategy of the countries, study participant and method difference.

Although the most common isolates in present study were *E. coli* (53.5) than *K. pneumoniae* (24.1%) the predominant ESBLs producer was *K. pneumonia* (78.6%) followed by *E. coli* (52.2%). This finding had agreement with previous studies done in Bahir Dar: 69.8% and 58.2% (64), in Jimma: 70.4% and 28.2% (65), and in Uganda: *K. pneumoniae* (72.7 %) and *E. coli* (58.1%) (63) which demonstrated predominance of ESBLs production by *K. pneumoniae* than *E. coli*. However, in other studies *E. coli* was the predominant ESBLs producer than *K. pneumoniae*; in Central India *E. coli* (50.14%) and *K. pneumoniae* (48.27%) (52), in Burkina Faso *E. coli* (67.5 %) and *K. pneumonia* (26 %) (71), in Adama, *E. coli* (51.5%) and *K. pneumonia* (11.5%) (66), in India *E. coli* (61.4%) and *K. pneumonia* (46.2%) (87).

In present study, the proportion of ESBLs producing *Enterobacteriaceae* among less than 15years children was 74.1% (100/135). This was in agreement with previous study done in Addis Ababa TASH 78.57% (67), in a Tertiary Care Hospital of North-West India 66.7% (94) and in rural Ghana 68% (95). However, this result was higher than study conducted in Burkina Faso (50.8%) (71).

7.4 Distribution of ESBLs producing *Enterobacteriaceae* in different specimens

In the present study, ESBLs producing *Enterobacteriaceae* was found predominantly in blood specimens 84.4% (76/90) (AOR = 3.22, 95% AOR = 1.38-7.53, $P < 0.05$) followed by wound or pus specimens 52.5% (21/40) and urine 50.7% (138/272). Other investigator also reported blood as major source of ESBLs producers: in Bahir Dar 84.8% in blood and 72.7% in open wound swabs (64), in Burkina Faso 75 % in blood (71), Iran 87.8% in blood and 48.5% in urine (72), in India by Hooja et al 79.2.0% in blood (94) and in India by Kumar et al 66.67% in blood and 54.67% in urine (96). This indicated that ESBLs producing *Enterobacteriaceae* have been becoming a serious problem in the treatment of invasive bacterial infections. However, in other studies urine was the major source of ESBLs producers; in Central India 52.28% in urine (52), in Uganda 64.9 % in urine and 47.4 % in pus (63), in Bangladish in urine 70.4% and in blood 16.5% (97). The difference might probably be attributed to study participant difference, risk factors or extent of antibiotics use.

7.5 Antibiotics susceptibility pattern of ESBLs producing *Enterobacteriaceae*

In this study, ESBLs producing isolates were found to be susceptible mainly to meropenem (96.7%) followed by amikacin (82.1%) and cefoxitin (70%); (P value < 0.05). This was in close agreement with study done in Ghana: meropenem (100%) (56), in Central India: meropenem (87.5%) and amikacin (83.92%) (52), in Jimma: amikacin (83.7%) (65), in India by Kaur, and his colleagues meropenem (94.0%) and amikacin (82.6%) (98), and indicating that these antibiotics were the most active treatment of choice for ESBLs producing *Enterobacteriaceae.*

In present study, the levels of co-resistance within different classes of antibiotics among the ESBLs isolates were significantly higher for most antibiotics tested. Of ESBLs producers, 63% were non-susceptible to gentamicin, 89.8% to trimethoprim-sulfamethoxazole, 69% to ciprofloxacin, 97.6% to cefepime and 91.5% to augumentin. Our result is comparable with the study conducted in Israel that showed 75% of ESBLs were non-susceptible to gentamicin, 70% to trimethoprim-sulfamethoxazole and 59% to ciprofloxacin (12), in Burkina Faso: 45% to trimethoprim-sulfamethoxazole, 89% to gentamicin, 80% to ciprofloxacin (71), in Ghana: 92.6% to trimethoprim-sulfamethoxazole, 91.2% to gentamicin and 41.1% to ciprofloxacin (56), in Nepal 90.7% to ciprofloxacin, 90.4% to trimethoprim-sulfamethoxazole, 63.12% to gentamicin (92) and in Central India: 50% to gentamicin, 87.5 % to ciprofloxacin and 94.6% to trimethoprim-sulfamethoxazole (52). These findings showed that ESBLs producing *Enterobacteriaceae* were the major cause of resistance to various antibiotics classes.

7.6 Comparison of double-disk synergy method against combination disk method

Detection of ESBLs producing by *Enterobacteriaceae* remains a challenge for microbiologists. Many clinical laboratories professionals may not be fully aware of the importance of organisms producing ESBLs and their method of detection. Failure to detect these enzymes has contributed to their uncontrolled spread and sometimes to therapeutic failures in patients who received inappropriate antibiotics and outbreaks of multi-drug resistant (70,99). ESBLs detection is of interest for infection control and for epidemiological purposes (100). We compared two phenotypic methods, Combination Disk Test (CDT) and Double Disk Synergy Test (DDST).

From ESBLs suspected isolate CDT identified 92.8% (246/265) isolates as ESBLs producers, where as the DDST identified 84.5% (224/265) by making synergy with one and/or two or three

cephalosporins. No *Enterobacteriaceae* isolate was found to be negative by CDT that was positive by DDST. Our finding was in agreement with a study conducted in Saudi Arabia: CDT (94%) and DDST (85%) (101), Ejaz H and his colleagues in Pakistan reported that CDT detected 99.5% while DDST detected 67.8% ESBLs producing isolate (102). A study conducted in Ahmadabad also reported somewhat lower than our finding that CDT detected ESBLs in 85.4% (41/48) and DDST in 75% (36/48) of the isolates (103), and in Saudi Arabia by Kader, A A and his colleagues 112/136 (82%) and 102/136 (75%) were positive for ESBLs by the DDST and CDT methods, respectively (104).

In the present study, from 224 ESBLs positivity by DDST, 5.8% (13) isolates showed synergy only with cefepime. Our finding was similar with study conducted in India by Kaur J and his colleagues reported 7.8% (17/218) (98), in Saudi Arabia by Kader and his colleagues found 10 (7.4 %) that showed a clear extension of the edge of inhibition by cefepime only (104). When we used DDST alone, from the total, 3.1% of ESBLs producing isolates were missed. The possible reason for missing may be the existence of chromosomally encoded or plasmid-encoded AmpC beta-lactamase enzyme that mask the synergistic effect of the clavulanic acid and the cephalosporin against ESBLs and may thus lead to false negative ESBLs test results (105).

Among the total 426 isolates, ESBLs producing *Enterobacteriaceae* was found to be 246 (57.7%) by CDT and 224 (52.6%) by DDST. These results were in good agreement with study done in India; Hooja S and his colleagues identified 60.2% by CDT and 56.8% by DDST (94) and Giriyapur RS and his colleagues identified 63.89% by CDT and 56.23% by DDST as ESBLs producers (106). This showed CDT was found to be better that DDST in the detection of ESBLs producing *Enterobacteriaceae.*

In the present study, as compared to CDT, DDST had a sensitivity of 91.1% (224/246), a specificity of 100% (19/19) and a positive predictive value of 100% (224/224) and negative predictive value of 46.3% (19/41). This finding was in agreement with study done by Leggese M et al., in sensitivity (90.9%), positive predictive value (95.2%) and negative predictive value (50%); but different in specificity (66.7%) (67). The difference in specificity may be the presence of AmpC beta-lactamase in our study was higher which may mask ESBLs and result in false negative in CDT, so that it might influence the specificity of DDST.

Although CDT had somewhat better detection performance, DDST might be the choice diagnostic where there is lack of combined antibiotics disks. In addition, instead of screening and confirming ESBLs production, one can perform directly the DDST along with routine antibiotic susceptibility testing. Performing ESBLs test routinely can help to report ESBLs production within 48 hours. The study showed that detection of ESBLs by DDST together with routine AST were important for infection control and timely treatment of patient with best alternative antibiotics.

Strength of the study

This is the first study done at different health facilities on magnitude of ESBLs producing *Enterobacteriaceae* in Addis Ababa, Ethiopia. This multi-center study revealed the extent of distribution of ESBLs among *Enterobacteriaceae* and degree of resistances to other non beta-lactam antibiotics. The magnitude of ESBLs in the city was done in relatively larger number of isolates than study done before.

Limitation of the study

- Although combinations of aminoglycosides, fluoroquinolones were tested, other beta-lactams and beta-lactamase inhibitors like piperacillin/tazobactam were not tested as it was beyond the scope of this study.
- We were unable to see status of the patients infected with ESBLs producing *Enterobacteriaceae.*
- Even though the isolates were collected from four bacteriology laboratories in Addis Ababa, the results cannot be applied to the entire city or country.
- The CDT method used as a reference standard to see the performance of DDST was not as good as polymerase chain reaction (PCR).

8 CONCLUSION

There was high prevalence of ESBLs producing *Enterobacteriaceae* and MDR isolates. The majority of ESBLs producing isolates were found primarily in blood and urine specimens. The most frequent ESBLs producing *Enterobacteriaceae* were *K. pneumoniae* and *E .coli*. Highest level of resistance to multiple classes of antibiotics was observed among ESBLs producers. The better option for treatment ESBLs producing *Enterobacteriaceae* are meropenem, amikacin and cefoxitin. ESBLs producing isolates showed high rate of resistance to ciprofloxacin, cefepime, cotrimoxazole and gentamicin as compared to non-ESBLs group. The performance of DDST was found to be very good when compared against CLSI recommended CDT.

9 RECOMMENDATIONS

- The high magnitude of ESBLs and MDR call for the strengthening and implementation of strong infection control strategies in all health facilities.
- Surveillance of antibiotics resistance and nationwide or hospital/community based study should be conducted in Ethiopia to know the countries prevalence of ESBLs by using both phenotypic and molecular techniques in conjunction with possible risk factors for the acquisition ESBLs and the outcome of the patient.
- The ever-increasing magnitude of ESBLs and MDR call for strengthening the clinical bacteriology research and diagnostic capacity of laboratory professionals for the detection and surveillance of antibiotic resistance.
- Carefully controlled prospective studies should be done to determine the preferable antibiotic for treatment of infection caused by ESBLs producing *Enterobacteriaceae.*
- Early detection of ESBLs producing *Enterobacteriaceae* in clinical laboratories is essential for the management of patients infected with ESBLs producing *Enterobacteriaceae*.
- If DDST can be performed in conjunction with the routine antibiotics susceptibility, it will help clinicians in selecting and prescribing proper antibiotics for treatment of infections caused by ESBLs producing and for the control of the dissemination of such bacteria.
- If a Laboratory detect ESBLs producing bacteria patient's report must state that the isolate is a suspected or confirmed ESBLs producer and also include a note stating that ESBLs production may predict therapeutic failure with penicillins, all cephalosporins (except cephamycins) and aztreonam, irrespective of their *in vitro* susceptibility.

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ANNEXES

Annex I: Trypticase Soya Broth (TSB) 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 the 30 gram of powder in 1litre 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.

Annex II: Preparation of MacConkey Agar

The medium is prepared from ready to use dehydrated powder, available from most suppliers of culture media. Its contents are Peptone, lactose, bile salts, sodium chloride, neutral red and agar.

The medium is usually used at a concentration of 5.2 g for 100 ml distilled water.

1. Prepare as instructed by the manufacturer. Sterilize by autoclaving at 121 ^{o}C for 15 minutes.
2. When the medium has cooled to 50–55 ^{o}C, mix well and dispense aseptically in sterile petri dishes. Date the medium and give it a batch number.
3. Store the plates at 2–8 ^{o}C preferably in plastic bags to prevent loss of moisture.
    - Shelf-life:Up to 4 weeks providing there is no change in the appearance of the medium to suggest contamination or an alteration of pH.
    - pH of medium should be within the range pH 7.2–7.6 at room temperature.

Annex III: Laboratory Procedure for Biochemical Tests, Inoculums Preparation, Inoculating Test Plates, Antimicrobial Discs Application and AST Interpretation

1. **Culture and Identification**

I. Bacteria growth from the stored isolate on MacConkey agar

Re-isolated □ Not re-isolated □

II. Identification steps for the isolated colonies

a) Colony Characteristics
b) Gram stain:______________
c) Oxidase test: positive □ /negative □
d) Lactose fermentation on MacConkey agar

☐ Lactose Fermenter ☐ Late Lactose fermenter ☐ Non lactose fermenter

III. Biochemical reactions

Identification of bacterial isolates involves the use of biochemical screening Medias. Indole, Urease, Mannitol, Triple sμgar iron (TSI), Citrate, Motility, Lysine Decarboxylase, Mannitol and Oxidase tests.

Lab ID:		Biochemical Reactions:							
		Indo	Urea	Man	TSI (k/k or a/a)	Cit	Mot	LDC	OX
Result	Positive								
	Negative								
Peripheral laboratory isolated bacteria______________________. EPHI bacteriology isolated bacteria_________________________.									

Key: LDC = Lysine decarboxylase, Man = Mannitol (mannite), Triple sμgar iron (TSI), Ox = Oxidase test, Cit = Citrate test, Mot = Motility, Ind = Indole test, Urea = Urease, H2S = Hydrogen sulphide (blackening), R = Red-pink (alkaline reaction), Y = Yellow (acid reaction),.

A. **Indole test:** Few colonies of the culture will be inoculated into peptone water and incubated at 37oC for 24 hours. Few drops of indicator (Kovac's reagent) will be added and gently shake to mix well. Colour change will be then observed. If the layer of indicator reagent turns to red within 1 minute, it is Indole positive (positive result). If the layer of indicator reagent remains yellow within 1 mi nute, it is indole negative (negative result).

B. **Urease test (Christensen's (modified) urea broth):** Urea agars will be inoculated heavily over the entire surfaces of the slants in bijou bottles. The cap will be loosened and then incubated at 37oC for 3-12 hours. A urease-positive culture produces an alkaline reaction in the medium, evidenced by pinkish red color of the Medium. Urease-negative organisms do not change the color of the medium, which is pale yellow-pink.

C. **Triple Sugar Iron (TSI) Agar Slant:** Using a sterile inoculating needle, stab the butt of the LIA slant twice then streak back and forth along the surface of the agar with the organism. Incubate at 37◦C for 18 to 24 h.
   - If acid slant–acid butt (yellow–yellow): glucose and sucrose and/or lactose fermented. If alkaline slant–acid butt (red–yellow): glucose fermented only.
   - If alkaline slant–alkaline butt (red–red): glucose not fermented. The presence of black precipitate (butt) indicates hydrogen sulfide production, and presence of splits or cracks with air bubbles indicates gas production.

B. **Citrate utilization test using Simmon's citrate agar:**Simmon's citrate slopes will be prepared in bijou bottles as recommended by the manufacturer (stored at 2-8oC). And the slopes will be then stabbed and incubated at 37oC aerobically for 48 hours. Blue colour indicates a positive reaction and if Simmon's citrate agar slopes remained as green in colour indicate negative reaction.

C. **Motility Test** (using motility agars): Motility agar will be prepared and inoculated with a straight inoculating needle making a single stab about 1-2cm down into the medium. The motility will be examined after 35-37oC for 24 hour. Motility will be indicated by the presence of diffuse growth (appearing as coloring of the medium) away from the line of inoculation.

D. **Lysine decarboxylase**: Decarboxylation of lysine can be detected by culturing bacteria in a medium containing the desired amino acid, glucose, and a pH indicator bromcresol purple. The acids produced by the bacteria from the fermentation of glucose will initially

lower the pH of the medium and cause the pH indicator to change from purple to yellow. The acid pH activates the enzyme that causes decarboxylation of lysine to amines and the subsequent neutralization of the medium. This results in another color change from yellow back to purple. Bacteria that decarboxylate lysine turn the medium purple. In addition bacteria that produce H2S appear as black colonies.

E. **Oxidase test**: A piece of filter paper is soaked with a few drops of oxidase reagent. A colony of the test organism is then smeared on the filter paper. Alternatively an oxidase reagent strip can be used. When the organism is oxidase-producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour.

2. Procedure for Performing the Disk Diffusion Test

I. Direct Colony Suspension Preparation

Prepare a saline suspension of the isolate from an overnight incubated agar plate (use a nonselective medium, such as blood agar) to obtain 0.5 McFarland turbidity (1.5 x 10^8cfu/ ml of the bacteria). For visual comparison, look through the suspension in transmitted light against a white background with contrasting black stripes.

II. Inoculating Test Plates

Fresh Müeller Hinton agar (MHA) plates will be used the same day or stored in a refrigerator (2-8ºC); if refrigerated, they should be wrapped in plastic to minimize evaporation. Just before use, if excess moisture is visible on the surface, plates should be placed in an incubator (35ºC) or, with lids ajar, in a laminar-flow hood at room temperature until the moisture evaporates (usually 10 to 30 minutes).

Müeller Hinton agar (MHA) plate will be inoculated within 15 minutes after the inoculum has been adjusted. A sterile cotton swab is dipped into the suspension, rotated several times, and gently pressed onto the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swab will then be streaked over the entire surface to the agar plate three times, with the plate rotated approximately 60° each time to ensure even distribution of the inoculum. A final sweep of the swab will be made around the agar rim. The lid may be left partly open for 3 to 5 minutes but no longer than 15 minutes to allow any excess surface moisture to be absorbed before the drug-impregnated discs are applied.

III. Antimicrobial Discs Application

Application of antimicrobial discs to an agar plate should be done within 15 minutes of inoculation of plates. The selected antimicrobial discs will be dispensed evenly onto the agar plate with the help of a forceps/sterile needle/surgical blade. Flame the tips of the applicator intermittently. Each disc must be pressed down to ensure complete contact with the agar surface.

1. Apply 12 discs on a 150 mm plate or 5 discs on a 100 mm plate, keeping at least a distance of 24 mm between discs. Dispensing too near to the edge of the plate should be avoided. Because some of the drugs diffuse instantaneously, a disc should not be relocated once it has come in contact with the agar surface.
2. Place discs that give predictably small zones like aminoglycosides, next to those discs that give larger zones like cephalosporins.
3. Disc containers should be removed from the refrigerator or freezer one to two hours before use, so they may equilibrate to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts cold disks.
4. Only those discs that have not reached the manufacturer's expiration date stated on the label will be used. Unused discs will be discarded on the expiration date.
5. Incubation– No longer than 15 minutes after discs are applied, the plates will be inverted and incubated at 35° ± 2℃ in ambient air.

IV. Preparation of combination disks

- The combination disks (indicator cephalosporin/clavulinic acid) were prepared according to CLSI recommendation. CLSI suggests making indicator disks with clavulinic acid by adding 10 µl of a 1000 µg/ml stock solution of clavulanic acid to cefotaxime and ceftazidime disks each day of testing or stored at -70 ^{o}C. Kotassium clavulanate analytical standard powder (Sigma-Aldrich Corp, St. Louis, MO USA) was used to prepare stock solution of clavulanate by dissolving 1000 µg of the powder in 1000µl of sterile distilled water.

V. Interpretation and Reporting of AST Results

Each plate was examined according to the recommendation of CLSI after overnight incubation (16-18 hour), for confluent growth and circular zones of inhibition. The diameters of the zones of complete inhibition, including the diameter of the disc, were measured to the nearest whole millimeter with callipers or a ruler. The measuring device is held on the back of the inverted petri dish, which is illuminated with reflected light located a few inches above a black, nonreflecting background. Zone margin should be considered the area showing no obvious visible growth detectable with the unaided eye. Faint growth of tiny colonies visible only by lens should be ignored. In case of presence of discrete colonies within clear zone of inhibition, repeat test with a subculture of a single colony/pure culture from the primary culture plate. If discrete colonies still appear, inner colony free zone size will be measured. For Proteus spp., swarming should be ignored.

VI. AST Result of the isolate at EPHI bacteriology laboratory:

S.No	Antimicrobial Agent	Disk content	Zone diameter nearest whole mm			Result of AST (S/I/R)/(Zone diameter in mm)	Comments
			R	I	S		
1.	Gentamicin	10 µg	≤ 12	13-14	≥ 15		
2.	Amikacin	30 µg	≤ 14	15-16	≥ 17		
3.	Amoxicillin+clavulanic acid	20/10 µg	≤ 13	14-17	≥ 18		
4.	Cefotaxime	30 µg	≤ 22	23-25	≥ 26		
5.	Ceftazidime	30 µg	≤ 17	18-20	≥ 21		
6.	Cefixitin	30 µg	≤ 14	15–17	≥ 18		
7.	Cefepime	30 µg	≤ 19	SDD19–24	≥ 25		
8.	Meropenem	10 µg	≤ 19	20-22	≥ 23		
9.	Ciprofloxacin(for Enterobacteria)	5 µg	≤ 15	16-20	≥ 21		
10.	Norfloxacin	10 µg	≤ 12	13-16	≥ 17		
11.	Trimethoprim+Sulfamethoxazole	1.25/23.75	≤ 10	11-15	≥ 16		

Annex IV: 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 V: Data Collection Form for *Enterobacteriaceae* isolates

1. Isolate identification laboratory:________________________
2. Patient referring Hospital:_____________________________ Inpatient/Outpatient
3. Age _______ Sex _______
4. Identification number of the isolate at site:_____________________
5. Identification number of the isolate for the study:_____________________
6. Isolated Enterobacteriaceae spps:
    - 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. Other:________
7. Type specimen:__________________________
8. Can the laboratory perform ESBL test? YES___ NO_____
9. If YES which method they use?

    A. DDST B. DDT C. IF AUTOMATED, NAME OF MACHINE_________

10. Result of AST pattern of the isolate at peripheral laboratory:

S.No	Antimicrobial Agent	Disk content	Zone diameter nearest whole mm			Result of AST (S/I/R)/(Zone diameter in mm)	Comments
			R	I	S		
1.	Ampicilin	10 µg	≤ 13	14-16	≥ 17		
2.	Gentamicin	10 µg	≤ 12	13-14	≥ 15		
3.	Tobramycin	10 µg	≤ 12	13-14	≥ 15		
4.	Amikacin	30 µg	≤ 14	15-16	≥ 17		
5.	Amoxicillin+clavulanic acid	20/10 µg	≤ 13	14-17	≥ 18		
6.	Cefuroxime	30 µg	≤ 14	15-17	≥ 18		
7.	Cefotaxime	30 µg	≤ 22	23-25	≥ 26		
8.	Ceftriaxone	30 µg	≤ 19	20-22	≥ 23		
9.	Ceftazidime	30 µg	≤ 17	18-20	≥ 21		
10.	Meropenem	10 µg	≤ 19	20-22	≥ 23		
11.	Ciprofloxacin (breakpoint for non-Salmonella)	5 µg	≤ 15	16-20	≥ 21		
12.	Norfloxacin	10 µg	≤ 12	13-16	≥ 17		
13.	Nalidixic acid	30 µg	≤ 13	14-18	≥ 19		
14.	Trimethoprim	5 µg	≤ 10	11-15	≥ 16		
15.	Trimethoprim+Sulfamethoxazole	1.25/23.75	≤ 10	11-15	≥ 16		
16.	Aztreonam	30 µg	≤ 17	18-20	≥ 21		
17.	Chloramphenicol	30 µg	≤ 12	13-17	≥ 18		
18.	Nitrofurantoin	300 µg	≤ 14	15-16	≥ 17		

Annex VI: Data Collection Form-1 at EPHI bacteriology laboratory

Lab ID	Age	Sex	Site	Type of Specimen	Isolate	CTX	CAZ	FEP	FOX	MER	SXT	CPR	GN	AMK	AMC	BETALACTAMASE TEST RESULT	

Annex VI: Registration form for final ESBLs result

Lab ID	Age	Sex	Specimen	Isolate	A	B	C		D	E	F	D-A	E-B	F-C	BETALACTAMASE TEST RESULT	
					CTX	CAZ	CFP	FOX	CTX/CA	CAZ/CA	CFP/CA				CDT	DDST

Annex VIII: Declaration

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

Name of the student: Dejenie Shiferaw Teklu

Signature: ____

Date: ____

Approval of the primary Advisor

Name of the primary advisor: Kassu Desta

Signature: ____

Date: ____

Approval of the Co-Advisor

Name of the co-dvisor: Mr. Melesse Hailu

Signature: ____

Date: ____

Approval of the Co-Advisor

Name of the co-dvisor: Mr. Abebe Assefa

Signature: ____

Date: ____