ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCE, SCHOOL OF MEDICINE

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY & PARASITOLOGY

Magnitude of Extended Spectrum Beta-lactamase producing Multidrug resistant *Enterobacteriaceae* isolates from patients attending Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia

By: Aminu Seman (BSc, MSc candidate)

A research thesis submitted to the graduate studies of Addis Ababa University, College of Health Sciences, School of Medicine, Department of Microbiology, Immunology and Parasitology, in partial fulfillment of the requirements for the degree of Master Sciences in Medical Microbiology

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List of abbreviations/Acronyms

AAERC	AHRI alert ethical review committee
AAU	Addis Ababa University
AHRI	Armauer Hansen research institute
ATCC	American type culture collection
AST	Antimicrobial susceptibility testing
CDT	Combined disk test
CLSI	Clinical and laboratory standard institute
CTX-M	Cefotaximase
DDST	Double disk synergy test
DNA	Deoxyribonucleic acid
DRERC	Department research and ethical review committee
ESBL	Extended spectrum beta lactamase
ESBL-PE-	Extended spectrum beta lactamase- producing Enterobacteriaceae
GNB	Gram negative bacteria
HAI	Hospital Acquired Infection
ICU	Intensive care unit
MDR	Multidrug resistant
OXA	Oxacillinase
PCR	Polymerase chain reaction
SHV	Sulphydril variable
SOP	Standard operating procedures
SPSS	Statistical Package for Social Study
TASH	Tikur Anbessa Specialized Hospital
UTI	Urinary tract infection
TEM	Temoneria
VEB-	Vietnam extended beta lactamase
WHO	World Health Organization

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Abstract

Introduction: Multidrug resistant (MDR) bacteria constitute a global emergency. Of which extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* (ESBL-PE) have become the most common type. ESBL-PE are important causes of serious infections in the community as well as in hospital settings.

Objective: To determine the magnitude and patterns of resistance of extended Spectrum β-lactamase producing *Enterobacteriaceae* isolates from patients attending Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia.

Methods: A cross sectional study was carried out to identify and characterize antimicrobial susceptibility pattern of *Enterobacteriaceae* isolates from TASH between July 2018 to November 2019. A total of 287 *Enterobacteriaceae* isolates were isolated from different specimens: blood, urine, wound site discharge, body fluids were analyzed using blood agar, MacConkey, Xylose lysine deoxycholate and salmonella shigella agar(SSA). Bacterial species identification was carried out using standard biochemical tests. Antimicrobial susceptibility testing and detection of ESBL production was performed using standard antibiotics on Muller Hinton agar using disk diffusion method and a combination disc method following 2018 CLSI guideline.

Results: The overall magnitude of ESBL production was 62.7% (n=180/287). Female had higher proportion of ESBL positivity (66.1%, n=84/127) than male patients (60%, n=96/160). Among the *Enterobacteriaceae* isolates tested, 81 (72.3%) of the 112 *K. pneumoniae*, 21 (65.5%) of 32 *K. oxytoca* and 61 (58.7%) of 104 *E.coli* were ESBL producers. Comparable ESBL production by 158 (64.5 %) of 245 isolates from in patients and 22 (52.4%) of 42 isolates from outpatient was observed. There was no significant association between hospitalization and ESBL production (P=0.136, OR=0.606, 95 % CI). However, when we compare ESBL production of isolates from different wards a high ESBL production was noted among isolates from pediatrics ward 57(31.7%) of 180 isolates. The ESBL producing isolates showed highest and lowest level of resistance to ampicillin and amikacin 96.9% and 9.0 %, respectively. Two-hundred sixty four (91.9%) of the 287 isolates had MDR pattern.

Conclusion: Significantly all isolates of *Enterobacteriaceae* showed higher rate of resistance to all tested antibiotics. But amikacin was found most effective for ESBL producers and non-producers. High rate of ESBL and high numbers of MDR organisms were isolated among *Enterobacteriaceae*. Therefore, strengthening of monitoring antibiotics resistance in addition to regulation of the spread of resistance genes and preparing strong policies for monitoring utilization of antibiotics is required.

Keywords: *Enterobacteriaceae*, Extended spectrum Beta-lactamase, Multidrug Resistant (MDR

Chapter I: Introduction

1.1 Introduction

Enterobacteriaceae are gram-negative bacilli, which are usually resident in the gastrointestinal tract. This group of gram negative bacteria is recognized as major causes of both nosocomial and community-acquired infections. Beta-lactams (mainly expanded-spectrum cephalosporins and carbapenems) and fluoroquinolones constitute the main therapeutic choices to treat infections caused by these microorganisms (Thenmozhi *et al*., 2014; Garza-Gonzalez *et al*., 2011).

The introduction of Beta-lactam antibiotics into the health care system during the latter period of the World War II was the most important achievement in medical science. However, since the introduction of antibiotic agents resistance has emerged and resulted in impaired treatment of human diseases (Forssten, 2009).

Antibiotic resistance may be intrinsic or acquired. Intrinsic means the microorganism is by definition resistant against a certain antibiotic naturally. Acquired refers to resistance that is a consequence of mutational events or gene acquisition via horizontal gene transfer (Georgios *et al*., 2014).

Acquired resistance to expanded-spectrum cephalosporins is mainly mediated by extended-spectrum beta-lactamases (ESBLs) that confer bacterial resistance to all beta-lactams except carbapenems and cephamycins, which arises mainly due to mutations in beta-lactamases encoded by the bla_{SHV}, bla_{TEM}, and bla_{CTX-M} genes (Leylabadlo *et al*., 2017; Harwalkar *et al*., 2013; Georgios *et al*., 2014). The most frequently detected and clinically important ESBLs belong to the TEM, SHV and CTX-M families (Nijhuis *et al*., 2012; Garza-Gonzalez *et al*., 2011).

ESBL has generally been defined as transmissible Beta-lactamases that can be inhibited by clavulanic acid, tazobactam or sulbactam, and which are encoded by genes that can be exchanged between bacteria (Shaikh *et al*., 2015). ESBLs are considered to be one of the most important antibiotic resistance mechanisms of gram negative bacteria (Ehlers *et al*., 2009). Therefore, this study was planned to determine the magnitude of ESBL producing multidrug resistant *Enterobacteriaceae* in Tikur Anbessa Specialized Hospital.

1.2 Literature review

1.2.1 Microbiology of *Enterobacteriaceae*

The *Enterobacteriaceae* are the largest and diverse family of Gram-negative rods, members of which are both free living in nature and part of the indigenous flora of humans and animals. A

few are adapted strictly to living in humans. *Enterobacteriaceae* grow rapidly under aerobic or anaerobic conditions (facultative anaerobes) on a variety of nonselective (e.g., blood agar) and selective (e.g., MacConkey agar) media and are metabolically active. These organisms do not form spores or demonstrate acid fastness. Members of this family share a similar cell wall, cell membrane, and internal structures morphologically. They can grow readily on simple media, often with only a single carbon energy source.

The family includes many genera (*Escherichia*, *Shigella*, *Salmonella*, *Enterobacter*, *Klebsiella*, *Serratia*, *Proteus*, and others), ferment a wide range of carbohydrates, possess a complex antigenic structure, and produce a variety of toxins and other virulence factors. These genera have been classified based on biochemical properties, antigenic structure, DNA–DNA hybridization, and 16S rRNA sequencing. These bacteria cause a variety of human diseases, including one third of all bacteremia, more than 70% of urinary tract infections (UTIs), and many intestinal infections (Carroll *et al*., 2015; Ray and Ryan, 2010).

1.2.2. Antimicrobial resistance in *Enterobacteriaceae*

Many antimicrobial drugs that were once effective against bacterial infections are now ineffective as a result of the evolution of antibiotic resistance through horizontal gene transfer and/or spontaneous mutations. These genetic changes render drugs ineffective by several mechanisms such as pumping out drug molecules by efflux pumps, altering drugs' target enzymes, and degrading or modifying drug molecules by producing enzymes e.g Beta-lactamases which is capable of inactivating beta-lactam antibiotics by splitting the amide bond of beta-lactam ring. Beta-lactamase production is the main mechanism of beta-lactam resistance in *Enterobacteriaceae* (Oz *et al*., 2014; Thenmozhi *et al.*, 2014).

Prolonged exposure of bacteria to a variety of Beta-lactams antibiotics has led to continuous mutation and production of Beta-lactamases and expanding their activity towards the newly developed Beta-lactam antibiotics. These enzymes are known as Extended-Spectrum Beta-Lactamases (ESBLs) (She *et al*., 2015).

ESBLs are a group of plasmid-mediated, diverse, complex and rapidly evolving beta lactamase enzymes predominantly described *in Enterobacteriaceae* and encoded by genes that can be exchanged between bacteria (Sharma *et al*., 2013). ESBLs are capable of conferring bacterial resistance to the penicillins, first, second and third generation cephalosporins, and aztreonam (Paterson and Bonomo, 2005). And they are also able to hydrolyze fourth generation

cephalosporin (Sharma *et al*., 2013), but not cephamycins and carbapenems. These enzymes are inhibited by Beta-lactamase inhibitors (clavulanic acid and sulbactam) (Singh *et al*., 2016).

On genetic grounds, the presence of ESBLs is associated with resistance to other classes of non–beta-lactam antibiotics (Nobrega and Brocchi, 2014). ESBLs together with the chromosomally encoded class C cephalosporinases (AmpC) constitute today's predominant resistance mechanism of gram-negative bacteria that inactivate Beta-lactam antibiotics by hydrolysis, which results in ineffective compounds (Stürenburg and Mack, 2003; Pitout and Laupland, 2008).

ESBLs are primarily produced by the *Enterobacteriaceae* family of Gram-negative bacteria, in particular *Klebsiella pneumoniae (K. pneumoniae*) and *Escherichia coli (E.coli)* (Sullivan and Schaus, 2015). And less commonly, produced by other members of *Enterobacteriaceae* family (*Enterobacter*, *Citrobacter*, *Proteus, Salmonella* and *Serratia*) (Nedjai *et al*., 2012; Yadav and Chauhan, 2016) and non-lactose fermenter Gram-negative bacteria, such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Dhillon and Clark, 2012).

1.2.3. Classification of beta-lactamases

Beta-lactamases are most commonly classified according to the Ambler molecular classification scheme and the Bush-Jacoby-Medieros functional classification system (Paterson and Bonomo, 2005).

The Ambler classification scheme is the original method of Beta-lactamase categorization which orders the enzymes into 4 classes (A, B, C, and D) based on molecular structure (Dhillon and Clark, 2012). The Beta-lactamase classes A, C, and D enzymes are serine Beta-lactamases; which have serine as the main catalytic residue in their active site. Whereas class B enzymes are Metallo Beta-lactamases which have heavy metals (Zn^{2+}) acting as the main catalytic agent on their active sites (Paterson and Bonomo, 2005; Stürenburg and Mack, 2003).

The Bush's scheme classifies Beta-lactamases according to the substrate profile, inhibitor profiles, and physical characteristics such as molecular weight and isoelectric point (Rodrigues *et al*.,2014). In this classification scheme there are four main groups and multiple subgroups; which are providing more immediate relevance to the physician or microbiologist in a diagnostic laboratory because the classification considers Beta-lactamase inhibitors and Beta-lactam substrates that are clinically relevant (Paterson and Bonomo, 2005) (Table 1.1)

Table 1.1: Classification of beta-lactamase

Bush-Jacoby-Medeiros Group	Molecular class (Ambler)	Distinctive substrate(s)	Representative enzymes	Defining characteristic(s)	References
1	C	Cephalosporins	*E. coli* AmpC, ACT-1, CMY-2, FOX-1, MIR-1	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes Cephamycins	(Padmini *et al*., 2017)
2b	A	Penicillins, early cephalosporins	TEM-1, TEM-2, SHV-1	Similar hydrolysis of benzylpenicillin and cephalosporins	(Ghafourian *et al*., 2014 and Padmini *et al*., 2017)
2be	A	Extended-spectrum cephalosporins, monobactams	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1	Increased hydrolysis of oxyimino- Beta-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	(Padmini *et al*., 2017)
2d	D	Cloxacillin,Extended-spectrum cephalosporins and Carbapenems	OXA-1, OXA-10,OXA-11, OXA-15, OXA-23, OXA-48	Increased hydrolysis of cloxacillin,Oxacillin and carbapenems	(Thenmozhi *et al.*, 2014)
3	B	Carbapenems	IMP-1, VIM-1, CcrA, IND-1,L1, CAU-1,GOB-1, FEZ-1, CphA, Sfh-1	Broad-spectrum hydrolysis including carbapenems but not monobactams	(Dhillon and Clark, 2012 ; Folgori *et al*., 2017)
4	Unknown	Miscellaneous enzymes that do not fit into other groups			(Ghafourian *et al*., 2014)

1.2.4. Evolution and dissemination of ESBLs

Beta-lactamases may be chromosomally encoded or plasmid mediated. The chromosomal enzymes are believed to have evolved from pencillin binding proteins (PBPs) by undergoing a number of mutations in order to escape the selective pressure exerted by Beta-lactam antibiotics (Rawat and Nair, 2010). Whereas plasmid mediated Extended spectrum Beta-lactamases are most often derived from mutation in the older Beta-lactamases (SHV-1, TEM-1 and TEM-2, CTX-M and OXA Beta-lactamases) (Yadav and Chauhan, 2016).

For the global dissemination of most common ESBL genes, mobile genetic elements like, Insertion sequences, integrons and transposons has played a crucial role since they can transfer promiscuously between bacteria (Espinar *et al*., 2015)

These genes are sporadically described all over the world. The global dissemination has occurred as two successive waves. The first included dissemination of strains producing TEM and SHV-derived Beta-lactamases. These ESBL-PE mostly belonged to the *Klebsiella* and *Enterobacter* genus and spread almost exclusively within hospitals. During the 1980s and 1990s, they caused small outbreaks relatively easily contained by infection control measures. The second wave involves CTX-M-type ESBL-PE, which mainly differ by two features. First, CTX-M ESBLs are mostly seen in the *Escherichia coli* species, probably because of the remarkable fit between this species and the type of plasmid. Second, the spread was not restricted to hospitals but occurred also in community settings (Espinar *et al*., 2015; Woerther *et al*., 2017; Pishtiwan and Khadija, 2019). TEM- and SHV-type Beta-lactamases, mainly produced by *K.pneumoniae*, have spread throughout hospital settings, and CTX-M enzymes, mainly produced by *E. coli*, have become predominant in the community (Hijazi *et al*., 2016).

1.2.5. ESBL Types

A) TEM-type ESBLs

The TEM-type ESBL are the most widespread plasmid-encoded serine Beta-lactamases which are derivatives of TEM-1 and TEM-2. TEM-1 was first reported in 1965 from an *E. coli* isolate from a patient in Greece, named Temoneira (hence the designation TEM) (Paterson and Bonomo, 2005). TEM-1 is the most commonly encountered Beta-lactamase in Gram negative bacteria which is responsible for up to 90% of ampicillin resistance in *E.coli* isolates and is also responsible for ampicillin and penicillin resistance in *H.influenzae* and *N. gonorrhoeae* (Thenmozhi et al., 2014).

TEM-2 was the first variant which differed from TEM-1 through the substitution of a lysine for a glutamine at position 39. However, TEM-2 is not considered an ESBL as the substrate profile is identical to TEM-1(Rupp and Fey, 2003). Both are able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and have negligible activity against extended-spectrum cephalosporins, and they are inhibited by clavulanic acid (Paterson and Bonomo, 2005).

Mutations within the $bla_{\text{TEM-1}}$ structural gene, apparently through antibacterial pressure, have allowed the enzyme to expand the hydrolysis capabilities to extended spectrum cephalosporin and Aztreonam while maintaining its original hydrolysis capabilities (Rupp and Fey, 2003).

The first ESBL phenotype was discovered in *K.pneumoniae and* named TEM-3. Initially it was known as CTX-1, because of its activity against cefotaxime. TEM-3 is different from TEM-2, by a replacement of two amino acids. Thus far, there are more than 200 variants of TEM (Ghafourian *et al*., 2014, Sidjabat and Paterson, 2015). Each TEM-derivative has a slightly different substrate profile in which one ESBL may hydrolyse a specific extended-spectrum cephalosporin more efficiently than another ESBL (Rubin and Pitout, 2014). Although TEM-type Beta-lactumases are most often found in *E.coli* and *K.pneumoniae* they are also found in other species of Gram-negative bacteria like, *E.aerogenes, M.morganii, P. mirabilis, P.rettgeri, P.aeruginosa,* and *Salmonella* spp (Rezai *et al*., 2015;Thenmozhi *et al*., 2014).

B) SHV type ESBLs

The first *bla*SHV-1 gene was identified in the 1970s in *E.coli* which have proved its activity against penicillins and first generation cephalosporin. The designation SHV refers to sulf*h*ydryl variable active site because it was thought that the inhibition of SHV activity by *p*-chloromercuribenzoate was substrate related and was variable according to the substrate used for the assay. SHV is part of the conjugative plasmid p453. The most likely ancestor of the plasmid-mediated SHV-1 is a chromosomal species-specific penicillinase detected in fecal *K.pneumoniae* isolates from neonates (Liakopoulos *et al*., 2016; Paterson and Bonomo, 2005).

The SHV type ESBL are more prevalent than the other types of ESBLs in clinical isolates of bacteria particularly in *K.pneumoniae* which is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species. Most strains have SHV type ESBL on their plasmid through the replacement of serine to glycine at position 238 and lysine to glutamate at position 240, while other less frequent but critical substitutions for the extended-spectrum phenotype occur on several amino acids including Ile8, Arg43, Glu64, Gly156, Asp179, and Arg205. The serine and lysine residues at position 238 and 240 are essential for hydrolyzing ceftazidime and cefotaxime respectively (Ghafourian *et al*., 2014; Bradford, 2001; Liakopoulos *et al*., 2016).

As of today, 189 SHV allelic variants have been described, having developed resistance to 3rd generation cephalosporin, monobactams and carbapenems, these variants have amino acid changes in fewer positions within the structural gene of SHV-1 (Liakopoulos *et al*., 2016, Bradford, 2001).

C) CTX-M type ESBLs

CTX-M type enzymes are a group of class A extended-spectrum Beta-lactamases that are rapidly spreading among *Enterobacteriaceae* worldwide. They have mainly been found in strains of *Salmonella enteric* serovar Typhimurium and *E. coli*, but have also been described in other species of *Enterobacteriaceae*. These enzymes were named for their greater activity against cefotaxime than other oxyimino-Beta-lactum substrates (e.g., ceftazidime, ceftriaxone, or cefipeme). These group of enzymes constitutes a rapidly growing cluster of enzymes encoded by transferrable plasmids. Today more than 150 CTX-M have been identified (Sidjabat and Paterson, 2015; Thenmozhi *et al*., 2014; Lahlaoui *et al*., 2014).

An important factor in their global dominance is the wide dissemination of bacterial clones producing CTX-M type ESBLs. For example, CTX-M-15 producing *E. coli* belonging to phylogenetic group B2 and sequence type (ST) 131 has been identified as the most prevalent in many countries (Calbo and Garau, 2015).

Contrary to other ESBL, the CTX-M family is a complex and non-homogeneous group of enzymes which can be categorized into five groups according to their amino acid sequence identities,CTX-M-1, CTX-M-2, CTX-M- 8, CTX-M-9, and CTX-M-25 and recently 2 additional groups were identified CTX-M-74 and CTX-M-75 (Shi *et al*., 2015; Lahlaoui *et al*., 2014).Within each branch, the identities generally range between 97 and 99%; however, the identity between the subgroups of CTX-M can be as low as 40% (Sidjabat and Paterson, 2015).

The original source of genes encoding CTX-M type Beta-lactamases are chromosomal genes resident in members of the genus *Kluyvera*, an environmental bacteria with little or no pathogenic activity against humans (Rossolini *et al*., 2008). Rather than arising by mutation, they represent examples of plasmid acquisition of Beta-lactumase genes (Thenmozhi *et al*., 2014). Multiple genetic mechanisms have apparently been involved in the capture and dissemination of CTX-M determinants. Genes encoding enzymes of several CTX-M groups (CTX-M-1, CTX-M-2, CTX-M-9 and CTX-M-25) have been found downstream of an ISEcp1 insertion sequence a transposable element that can co-mobilize flanking DNA fragments via a one-ended transposition mechanism (Rossolini *et al*., 2008).

D) OXA type ESBLs

OXA (oxacillin-hydrolyzing) Beta-lactumases were long recognized as a less common but also plasmid mediated class D Beta-lactamase variety which has been found mainly in *P. aeruginosa* but also in *Enterobacteriaceae* and In particular OXA-21 was found in a strain of *A. baumannii*. The OXA beta-lactamases contain active site serine groups, they usually confer resistance to amino- and ureidopenicillins. The OXA beta-lactamase family was originally created as a phenotypic rather than a genotypic group for a few beta-lactamases that had a specific hydrolysis profile. Therefore, there is as little as 20% sequence homology among some of the members of this family (Thenmozhi *et al*., 2014; Stürenburg and Mack, 2003; Bradford, 2001).

These beta-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D, functional group 2d and they are poorly inhibited by clavulanic acid. Unlike the TEM enzymes, this group had a heterogeneous substrate profile and was encoded by a much narrower range of plasmid located on Tn*21*derived transposon, inserted between the *aad* gene encoding aminoglycoside resistance and its promoter, had a lower specific activity against penicillin than the TEM beta-lactamases. Their emergence presumably coincided with the widespread introduction of flucloxacillin and methicillin for the treatment of staphylococcal infections (Bradford, 2001; Evans and Amyes, 2014; Thenmozhi *et al*., 2014).

With an exception of OXA-10 most OXA-type beta-lactamases do not hydrolyze the extended-spectrum cephalosporins to a significant degree and are not regarded as ESBLs. OXA-10 hydrolyzes (weakly) cefotaxime, ceftriaxone, and aztreonam, giving most organisms reduced susceptibility to these antibiotics. The other OXA type ESBLs in this group includes: OXA-11, -14, -16, -17, -19, -15, -18, -28, -31, -32, -35, and -45(Paterson and Bonomo, 2005). Several of the OXA-type ESBLs have been derived from OXA-10. The OXA-11 was the first example of an OXA enzyme that had become through mutation by changing two amino acids from the OXA-10 at position 143 arginine to serine and at 157 a glycine to aspartate. In addition to OXA-11, the OXA types -13, -14, -16, -17,-19, and -28 ESBLs have been derived from OXA-10. All of these ESBLs have been found in *P.aeruginosa,* which possess two to nine amino acid variations from the parental enzyme (Bradford, 2001; Evans and Amyes, 2014).

E) Other ESBLs

While the majority of ESBLs are derived from TEM or SHV beta-lactamases and others can be categorized with one of the newer families of ESBLs, a few ESBLs have been reported that are

not closely related to any of the established families of beta-lactamases. PER can be functionally classified into the group of Ambler's class A, share only around 25 to 27% homology with known TEM- and SHV-type ESBLs which efficiently hydrolyzes penicillins and cephalosporins and is susceptible to clavulanic acid inhibition, was first detected in *P.aeruginosa* and later in *Salmonella enteric* serovar Typhimurium and *Acinetobacter* isolates (Paterson and Bonomo, 2005; Saliu *et al*., 2017).

Another enzymes that are somewhat related to PER-1 are the VEB-1 beta-lactamase which was first found in a single isolate of *E. coli*, but was subsequently found in a *P. aeruginosa*, CME-1, from *Chryseobacteriummeningosepticum*,TLA-1, from *E.coli*. All of these beta-lactamases are related but shows only 40-50 % homology. These enzymes all confer resistance to oxyimino-cephalosporins, especially ceftazidime, and aztreonam (Bradford, 2001; Thenmozhi *et al*., 2014).

1.2.6. Epidemiology of ESBLs

For the first time ESBL phenomenon began in Western Europe, most likely because expanded-spectrum beta-lactam antibiotics were first used there clinically. Since then ESBL has been found all over the world as presented in Figure 1.1 and Table1.2. Rates of ESBL production was highest in *E.coli* and *K.pneumoniae.* In India 52.49% ESBL production was observed, and the major producer was *K.pneumoniae* (Sharma *et al*., 2013). In Nepal the rate of ESBL production is 40.6% with *Escherichia coli* isolates accounting for 46.5% of cases (Parajuli et al., 2016). Different study has showed that the rates of ESBL producers are dramatically increasing, in recent years different members of the *Enterobacteriaceae, Enterobacter* spp and *S. marcescens* (Nedjai *et al*., 2012), Shigella spp (Li *et al*., 2015), *K.oxytoca, C.freundii* (Fernandes *et al*., 2014), *P. mirabilis* (Andrew *et al*., 2017) and members of the non-fermenter group *P.aeruginosa* (Bokaeian *et al*., 2015) and *A. baumannii* (Alyamani *et al*., 2015) are emerging ESBL producers. In Ethiopia the rate of ESBL is variable across different regions in Harar 33% reported (Seid and Asrat, 2005); in Adama, 25% (Selassie, 2016); and in Bahr Dar, 57.6% (Abera *et al*., 2016).

Table 1.2: The magnitude of ESBL producing *Enterobacteriaceae* in the world

S.no	Year	Study area	Design	Sample size	%ESBL	Reference
1	2011- 2014	Mexico	CS	70	53	(Miranda-Romero *et al*., 2017)
2	2008-10	Portugal	CS	193	81.3	(Fernandes *et al*.,., 2014)
3	2011-2012	India	CS	722	52.49	(Sharma *et al*., 2013)
4	2003 -2007	Bangladesh	CS	339	11.8	(Lina *et al*., 2014)
5	2013-14	Iran	CS	262	28.6	(Peymani *et al*., 2017)
6	2012 -2014	Saudi Arabia	CS	107	94	(Alyamani *et al*., 2015)
7	2010–2012	Morocco	Retrospective	1472	4.5	(El bouamri *et al*., 2015)
8	2006-07	Madagascar	CS	909	26.3	(Rakotonirina *et al*., 2013)
9	2013- 2014	Ethiopia	CS	133	25	(Selassie, 2016)
10	2014	Ethiopia	CS	33	78.57	(Legese *et al*., 2017)
11	2014	Gondar	CS	384	14.8	(Engda *et al*., 2018)
12	2012	Addis Ababa	CS	267	51.2	(**Desta *et al*., 2016)**

Key; CS: cross-sectional

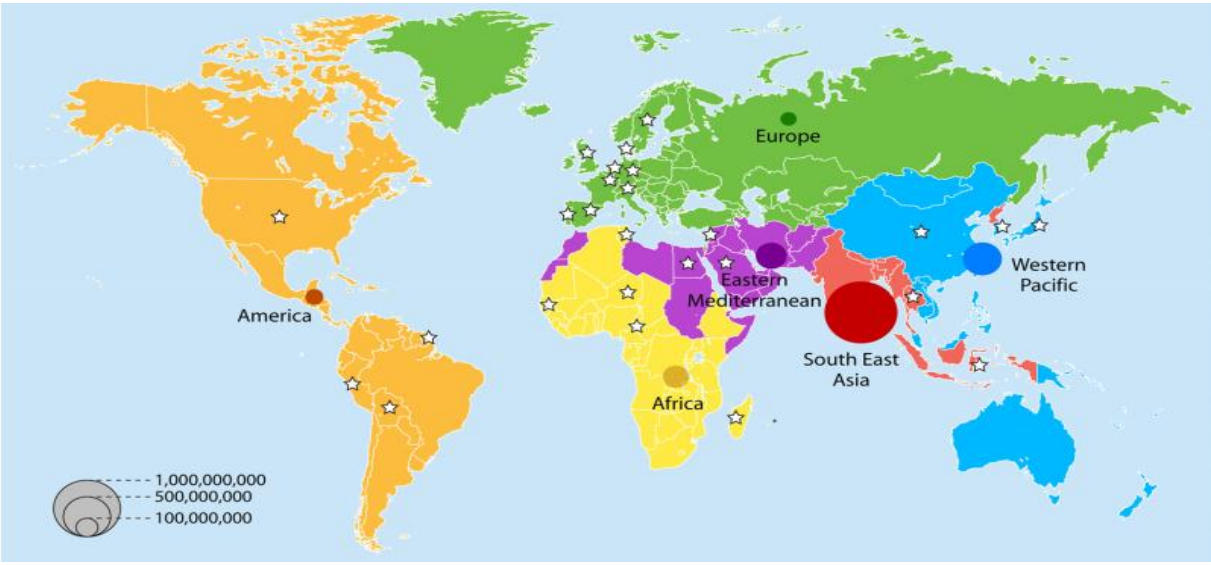

Figure 1.1: Number of ESBL carriers in the community in 2010, according to WHO region grouping. The 6 WHO regions are represented by different colors. WHO estimates of the population of each geographic area in 2010. Stars represent countries with available data for modeling. Each bubble area is proportional to the estimated number of ESBL carriers in that region *(*Woerther *et al.*, 2013*).*

1.2.7. Laboratory detection of ESBL

The increasing incidence of infection with *Enterobacteriaceae* producing emerging beta-lactamases, such as extended spectrum beta-lactamases (ESBL) has become a great concern. Infection by this ESBL producing multidrug resistant *Enterobacteriaceae are* associated with increased morbidity, mortality, treatment failure, longer hospital stay, rising healthcare costs and limited therapeutic options. Therefore, early detection and identification of these resistance enzymes will enable optimum antimicrobial therapy and ensure timely introduction of infection control procedures to prevent further spread (Slama, 2008; Willems *et al*., 2013). Early detection and identification of these enzymes can be done in two methods, **(i)** Phenotypic and **(ii)** genotypic methods. **(i)** Phenotypic methods use the principle that most common ESBLs (TEM, SHV, and CTX-M) are Beta-lactamase inhibitor susceptible and thus, this property is utilized for diagnosis. If the strain produces ESBL, 3rd generation cephalosporin combined with clavulanic acid inhibit bacterial growth more efficiently than the cephalosporin alone. This method has two steps, screening and confirmation steps. Phenotypic method is routinely used in clinical laboratory because it is easy to perform, convenient, and inexpensive, can be used with automatic machine such as VITEK-2. However, this method cannot verify the exact type of ESBLs (SHV, TEM, or CTX-M) (Forssten, S., 2009; Thirapanmethee, 2012).

Phenotypic screening methods is based on decreased susceptibility to extended-spectrum cephalosporin (cefotaxime, ceftazidime, ceftriaxone and cefopodoxime) in primary susceptibility testing and then confirmation by either combined-disk test(CDT) or double-disk synergy tests (DDST). The confirmation is by comparison of the inhibition zone around both cefotaxime and ceftazidime disks with or without clavulanate. Different combination or synergy of beta-lactamase inhibitor with various expanded spectrum cephalosporins and aztreonam have also been proposed (Garrec *et al*., 2011; Thirapanmethee, 2012; Poulou et al., 2014). But, the Clinical and Laboratory Standards Institute (CLSI) recommends a phenotypic confirmation by combined-disk test, clavulanate with 3rd generation cephalosporin. The result is confirmed positive when the inhibition zone difference is more than 5 mm (CLSI, 2018).

Genotypic detections are gold standard techniques, which uses molecular method to detect the ESBL encoded gene present in bacteria. The exact types of ESBLs can be identified by this method. The genotypic methods usually use the polymerase chain reaction (PCR) technique with oligonucleotide primers that are specific for a beta-lactamase gene. Oligonucleotide primers can

be chosen from sequences available in public databases such as Genbank. The PCR product will be subsequently sequenced for confirmation and classification of ESBL variants. By these methods it is possible to identify the different point mutations that can cause an ESBL activity. This method can detect the gene even in small amount, which cannot be detected by phenotypic detection. The advantage is that it can detect directly from specimen, not necessary to isolate pure culture (Forssten, S., 2009; Thirapanmethee, 2012)

1.3. Statement of the problem

Antibiotic resistance is a rapidly increasing global emergency of the 21st century that calls for action from all society, causing both communities acquired and nosocomial infections. Multidrug resistant (MDR) bacteria have spread worldwide with extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* (ESBL-PE) as the most prevalent type (Woerther *et al*., 2017; Singh *et al*., 2016; Moghaddam *et al*., 2017). Organisms that produce ESBLs remain an important reason for therapy failure with cephalosporins and have serious consequences for infection control. Infectious Diseases Society of America listed ESBL-producing *Klebsiella* spps and *E coli* as one of the six drug-resistant microbes to which new therapies are urgently needed (Pitout and Laupland, 2008; Sidjabat and Paterson, 2015). Beta-lactamase producing *Enterobacteriaceae* are commonly cross-resistant to other classes of antibiotics, such as fluoroquinolones, trimethoprim-sulfamethoxazole, and aminoglycosides, resulting in limited therapeutic options to treat infections caused by these pathogens (Sheng *et al*., 2013).

There have been a large number of ESBL outbreaks reported in the past decade in Africa, Asia, Europe, and North America, mostly originating in hospitals or in nursing homes, In the United States ESBL-producing *Enterobacteriaceae* causes 26,000 hospital associated infections (HAI) and 1,700 deaths per year in 2013. Some of these isolates were resistant to nearly all antibiotics in the penicillin and cephalosporin classes (Ventola, 2015).

ESBL-producing *Enterobacteriaceae* has been spreading at an alarming rate in Europe. Although there is extensive difference between European countries, almost every European country has experienced outbreaks with ESBL-producing organisms (Dhillon and Clark, 2012; Coque *et al*., 2008). ESBL-producing *Enterobacteriaceae* are also a large problem in African healthcare institutions and communities. The prevalence of ESBL-producing *Enterobacteriaceae* has been shown to vary between African countries and there is a trend of higher prevalence of ESBL in stool samples than in other specimens. There is also a trend of increasing prevalence over time in

some countries. In Tunisian for example ESBLs have increased from 11.7 to 77.8% from 2005 to 2010 among *K.pneumoniae* (Storberg, 2014). The emergence and transmission of Multidrug Resistant (MDR) bacteria are becoming major public health threats. Specially, bacteria that produce extended-spectrum beta-lactamases (ESBL) are of great concern because their resistance reduces considerably the treatment options (Ouedraogo *et al*., 2016). Being able to hydrolyze the majority of beta-lactam antibiotics that are currently in use, pose a serious clinical problem (Stürenburg and Mack, 2003). In addition, the coexistence of multiple ESBLs in same clinical strain including AmpC beta-lactamases, carbapenemases, and other antibiotic resistance plasmid determinants further creates the therapeutic confrontation (Parajuli *et al*., 2016).

In Ethiopia the burden of ESBL has increased from 62.8% in stool and blood samples (Storberg, 2014) to 78.57% in blood (Legese *et al*., 2017). However there is no generalized data concerning the magnitude of ESBL producing Multidrug resistant *Enterobacteriaceae* isolates, which are responsible for a number of clinically important diseases. By considering the existing gap, the current study is planned to determine the magnitude of ESBL producing MDR *Enterobacteriaceae* causing different site infection from hospitalized and out patients.

1.4. Significance of the study

Antibiotic resistance is an emerging problem and new strains which are highly drug resistant are being reported from different places of the world. This study has provided updated figures of ESBL producing multidrug resistant strains of *Enterobacteriaceae* among patients attending Tikur Anbessa Specialized Hospital. Knowing the magnitude is important for developing appropriate infection prevention and setting effective control measures to prevent the spread of this threat in the hospital and the community. And also this study has addressed the pattern of ESBL producing multidrug resistant *Enterobacteriaceae* in the different wards of the hospital which is essential to take appropriate measures on wards which were occupied by these resistant microorganisms. This study has also tried to figure out one of the most responsible factors resulting in treatment failure which was production of ESBL enzymes. Furthermore, the finding of this study will be used as current information on the area about the magnitude of ESBL producing MDR *Enterobacteriaceae.*

1.5. Objectives

General objective

To determine the magnitude and antimicrobial susceptibility pattern of ESBL producing multidrug resistant *Enterobacteriaceae* isolates in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia

Specific objectives

- Determine the magnitude of ESBL producing *Enterobacteriaceae* isolates from in and out patients attending TASH
- Determine the antimicrobial susceptibility pattern of ESBL producing isolates from in and out patients attending TASH
- To compare antimicrobial susceptibility pattern of ESBL producing and none producing isolates of *Enterobacteriaceae* from patients attending TASH
- Determine the magnitude of ESBL producing multidrug resistant *Enterobacteriaceae* isolates from patients attending TASH

CHAPTER II: Materials and Methods

2.1 Study area

The study was conducted in Tikur Anbessa Specialized Hospital (TASH), located in the capital city Addis Ababa. TASH is the largest specialized hospital in Ethiopia established in 1972, with over 800 beds and provides clinical services to 370,000-400,000 patients a year. TASH is affiliated with the Addis Ababa University's School of Medicine and serves as a training center for undergraduate and postgraduate medical students, dentists, nurses, midwives, pharmacists, medical laboratory technologists, radiology technologists, and others who shoulder the health problems of the community and the country at large. TASH is now the main teaching hospital for both clinical and preclinical training of most disciplines. It is also an institution where specialized clinical services like oncology and cardiac treatments that are not available in other public or private institutions are rendered to the whole nation. TASH has a well-furnished diagnostic laboratory with a number of departments like; Immunohematology, clinical chemistry, quality office, molecular biology and microbiology. The microbiology department has in it parasitology unit and bacteriology unit. This study was conducted at the bacteriology unit which process about eighty to one hundred clinical samples a day.

2.2 Study design and period

Institutional based cross sectional study was conducted from July 2018 to November 2019 in Tikur Anbessa Specialized Hospital, Addis Ababa Ethiopia.

2.3 Study Population

2.3.1. Source population

The source population of this study were all patients who were suspected for bacterial infections and indicated for diagnosis by culture and antimicrobial susceptibility tasting at diagnostic microbiology laboratory of TASH during the study period.

2.3.2. Study population

All patients who were confirmed for *Enterobacteriaceae* infections in diagnostic microbiology laboratory of TASH during the study period.

2.3.3. Sampling methods

Successive convenient sampling technique was applied

2.3.4 Sample size calculation

The sample size was calculated based on single-population proportion using a previous study done in the same area (Legese *et al*., 2017).

By using the formula, $n = \frac{Z^2 p(1-p)}{d^2}$, P=0.78 from previous study; Z = 1.96 (i.e., for a 95% C.I.) and d=0.05

This gives, 261

Therefore, we have collected a total of 287 isolates including 10% non-response rate.

2.4. Eligibility

Inclusion criteria

All patients with *Enterobacteriaceae* infections and willing to participate in the study

Exclusion criteria

Patients who were unwilling to consents or assents

2.5. Study variables

a) Dependent variables

- Antibiotic resistance pattern
- ESBL production
- Types of organism
- MDR pattern

b) Independent variables

- Socio demographic characteristics: Age, Sex and etc
- Ward type
- Sample type

2.6. Operational Definitions

Hospital acquired infections are those infections acquired in hospital or health care service unit that first appear 48 hours or more after hospital admission.

Multidrug-resistant: acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Shamsuzzaman, 2015)

2.7. Data collection techniques and tools

Participants were recruited after their clinical specimens become positive for *Enterobacteriaceae.* Patients who were hospitalized were approached when their clinical sample is being processed at bacteriology laboratory of TASH for the socio-demographic data whereas outpatients were traced during their days of appointment by discussing with the attending physician.

2.7.1. Demographic data collection

Demographic data of the patients who were positive for *Enterobacteriaceae* was recorded by using predesigned questionnaires and from medical records after obtaining informed consent or assent.

2.7.2. Bacteria isolation

Blood sample was delivered inoculated on BacT/ALERT® 3D culture bottles and incubated in automated BacT/ALERT® 3D machine at $35^{o}\pm2^{o}C$ in 5% CO_2 until five days. When a bacterial growth is there the machine gives a flag and an alarm, then subsequently subcultured on MacConkey agar (Mac, Oxoid UK), 5 % sheep blood agar plate (BAP, Oxoid UK) and chocolate agar plate (CHA, Oxoid UK) identification was made following WHO guideline for identification of gram-negative bacilli (Vandepitte *et al*., 2003). For those not flagged blood culture bottles to the end of the fifth day, blind subculture as of the flagged one and gram staining was performed and results were taken as negative when no evidence of bacteria was observed.

Urine sample was inoculated in to 5% sheep blood agar (BAP, Oxoid UK) and MacConkey agar plate (Mac, Oxoid UK). Wound site discharges and body fluids were inoculated into 5% sheep blood agar (BAP, Oxoid UK) and MacConkey agar plate (Mac, Oxoid UK). CSF samples were inoculated into 5% sheep blood agar (BAP, Oxoid UK), Chocolate agar plate and MacConkey agar plate (Mac, Oxoid UK) and stool specimens were inoculated in Salmonella-Shigella agar plates (SS agar, Oxoid UK) and Xylose Lysine Deoxycholate agar (XLD, Oxoid UK). All sample inoculated plates were incubated at 35°C $\pm$ 2^{o} C for overnight aerobically. All positive cultures were characterized by colony characteristics, Gram stain, and standard biochemical tests using the WHO guideline for identification of gram-negative bacilli (Vandepitte *et al*., 2003).
Gram negative bacteria were grouped in to lactose fermenter and non-lactose fermenter for easy identification by using different biochemical tests. The two groups were characterized to species levels using indole, urea, triple sugar iron, citrate, lysine decarboxylase, motility and Malonate tests. In the meantime pure colony was collected and stored at –80°C in Tryptic Soy broth with 20% glycerol till further tests (Ahmed *et al*., 2013).

2.7.3. Antimicrobial susceptibility testing (AST)

Using a sterile wire loop, pure colonies of a young culture was picked from blood agar and emulsified in nutrient broth to prepare a 0.5 McFarland turbidity standard using McFarland densitometer. From the 0.5 preparation we inoculated onto Muller-Hinton agar (MHA, Oxoid UK) by lawn method for the AST tests. The AST was performed using the Kirby–Bauer disc diffusion method following the recommendation of Clinical and Laboratory Standards Institute (CLSI., 2018) by using fifteen antibiotics namely, ampicillin (10 µg, BD USA), gentamicin (10 gµ, BD USA), amikacin(30 gµ, BD USA), tobramycin(30 µg, BD USA), cefotaxime(30 gµ , BD USA), ceftazidime (30 µg , BD USA), ceftriaxone(30µg , BD USA), ciprofloxacin (5 gµ , BD USA), trimethoprim/sulphamethoxazol (25 µg, BD USA), cefepime (30µg, BD USA), cefoxitin (30 µg , BD USA) , meropenem (10µg , BD USA), amoxicillin/clavulanic acid (20/10 µg , BD USA), pipracillin-tazobactam (100/10 µg , BD USA) and cefuroxime (30 µg , BD USA). After overnight incubation we measured the zone of inhibition as per recommendation of CLSI and interpreted as resistant, intermediate and sensitive (CLSI., 2018).

2.7.4. Screening test for ESBL

All *Enterobacteriaceae* strains that showed decreased susceptibility to ceftriaxone cefotaxime, ceftazidime by Kirby-Bauer disk diffusion method were considered for ESBL screening. If the zone of inhibition is < 22 mm for ceftazidime, <25 mm for ceftriaxone and < 27 for cefotaxime they were considered as potential ESBL strain and selected for further phenotypic confirmatory test as described below (CLSI., 2018).

2.7.5. Phenotypic confirmatory test for ESBL

ESBL phenotypic confirmatory tests were performed for strains that showed decreased susceptibility or resistance to ceftriaxone (30µg BD USA), cefotaxime (30µg BD USA) and/or ceftazidime(30µg BD USA). The suspected strains were inoculated onto Muller-Hinton agar (Oxoid). Antimicrobial disks cefotaxime (30µg BD USA), ceftazidime (30µg BD USA) and ceftriaxone (30µg BD USA) alone and then in combination with clavulanate (10µg) were placed at distance of 22 mm, center to center. Incubated at 35$\pm$ 2^{o}C for 16-18 hours, the difference in zone of inhibition was measured. A 5-mm increase in zone diameter for cefotaxime and ceftazidime tested in combination with clavulanate versus its zone when tested alone was considered indicative of ESBL production (i.e., the presence of a clavulanate effect) according to CLSI guideline (2018).

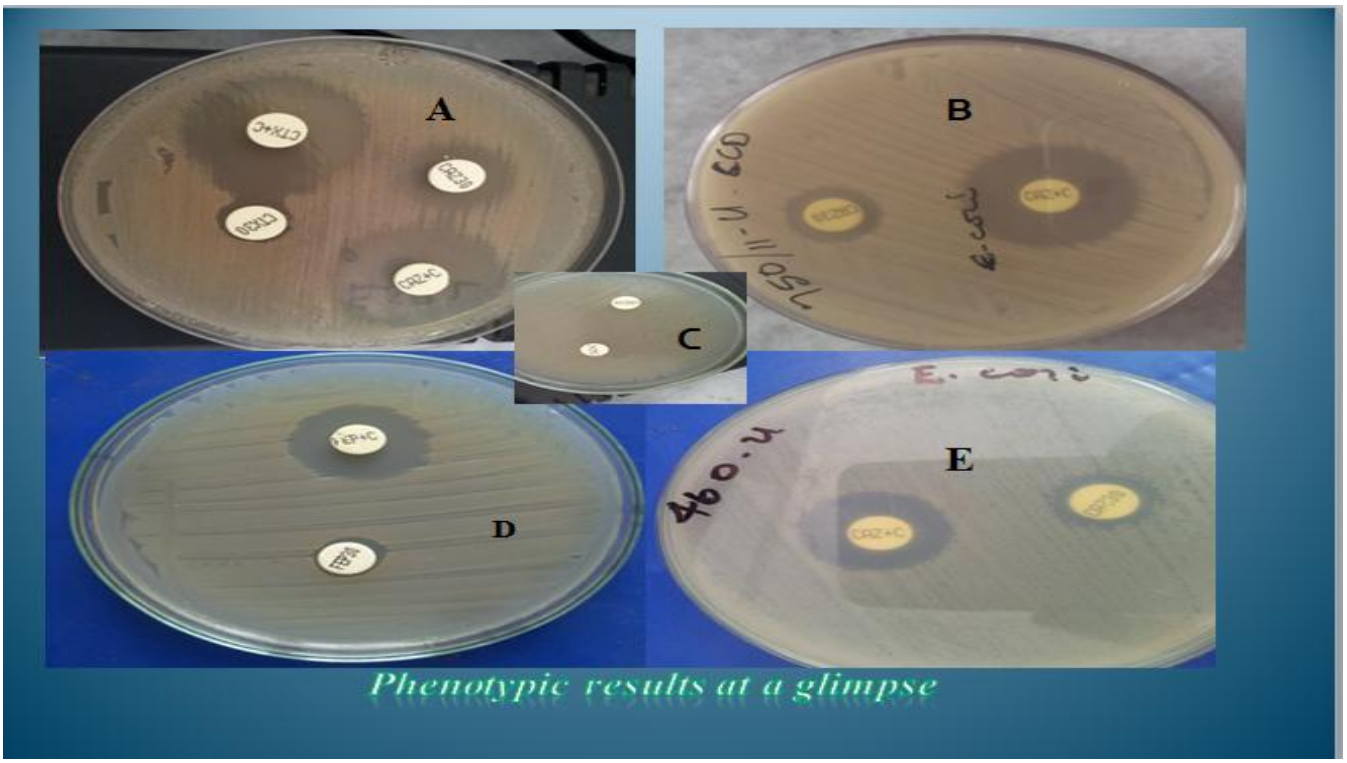

Figure 2.1: Some of phenotypic test results by using combined disc confirmation method. A) ESBL posetive *K.pneumoniae* by cefotaxime and ceftazidime disks alone and in combination with clavulanic acid, B) ESBL positive *E.coli* by cefotaxime(CTX)/cefotaxime+clavulanic acid (CTX+C), C) ESBL negative *K.oxytoca* isolates, D) ESBL positive *M.morgani* by cefipeme(FEP)/cefipeme+clavulanic acid (FEP+C) and E) ESBL posetive *E.coli* isolate by Ceftazidime(CAZ)/ceftazidime+clavulanic acid(CAZ+C). Note tests are interpreted as positve when there was an increase in diameter of inhibition by >5mm on disks containing clavulanic acid combination.

2.7.6. Work flow of laboratory activities

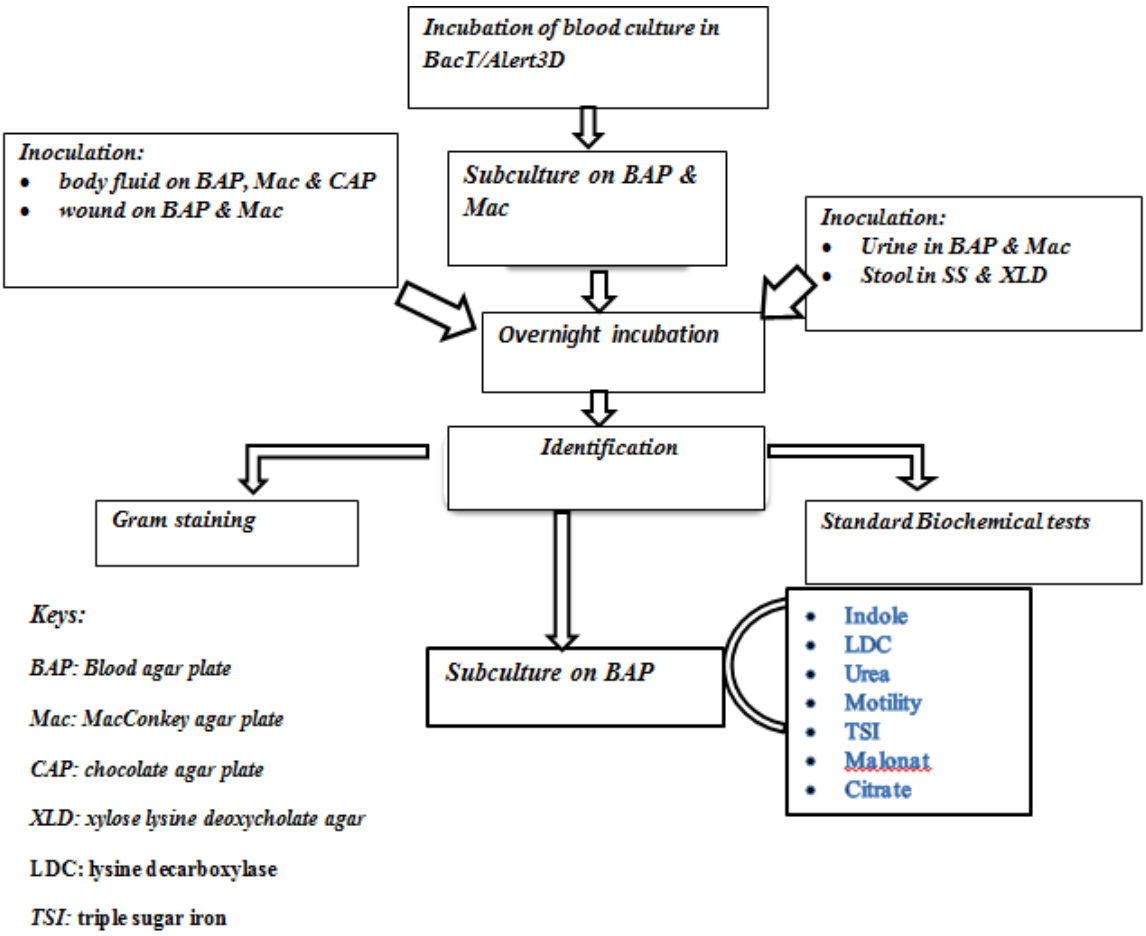

Figure 2.2: Laboratory identification of Enterobacteriaceae

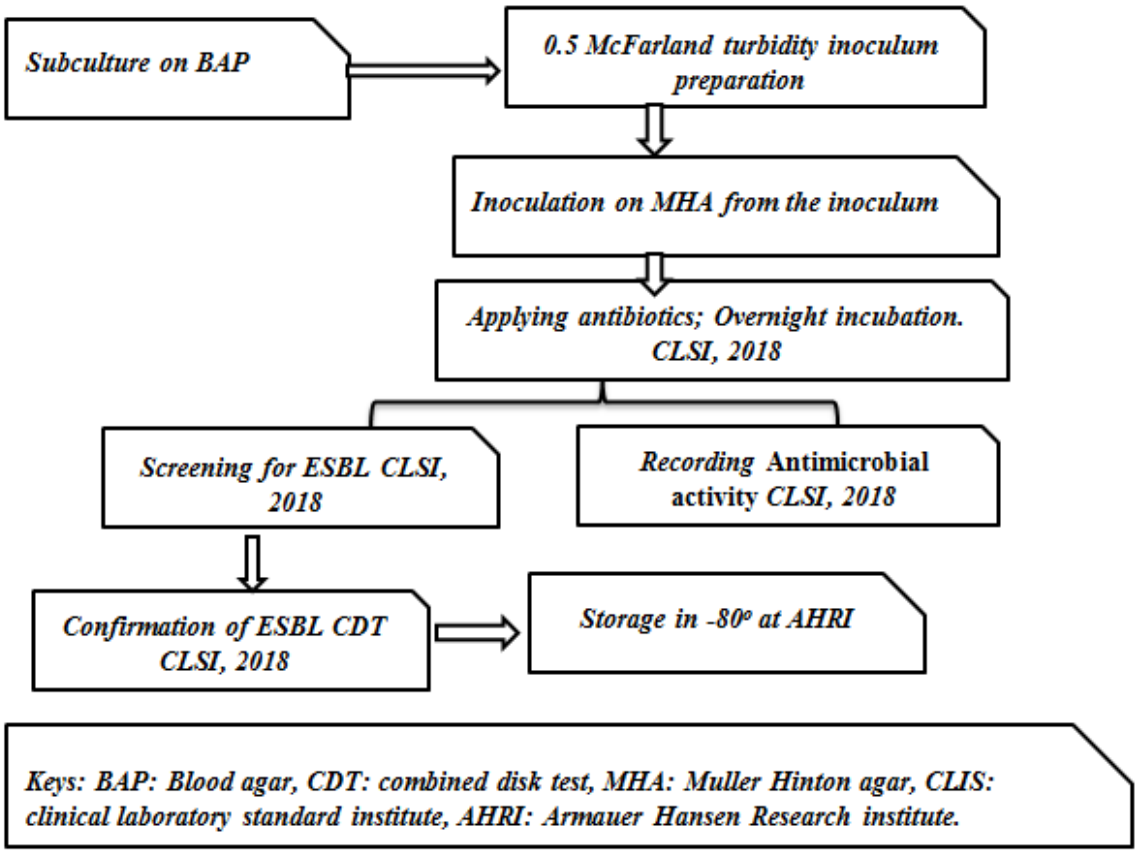

Figure 2.3: Laboratory activities for determining AST and ESBL pattern of *Enterobacteriaceae*

2.8. Quality assurance

The reliability of the study findings was guaranteed by implementing Quality control (QC) measure throughout the whole processes of the laboratory works. All materials and equipment were sterilized prior to the procedure. Standard operating procedures (SOPs) were applied during the three steps of laboratory work (pre-analytical, analytical and post-analytical) to evaluate and monitor the quality of the test result and to maintain high standard of accuracy of the result. Sample media from prepared were incubated at 37^{o} C for overnight to see if there is any contamination. The completeness and clearness of the questionnaires was assessed by doing a pre-test on selected participants. Data was Double entered to ensure the quality of output information. Any incomplete data has been disqualified from the study. Control strains *E. coli*

ATCC 25922 as ESBL negative and *K.pneumoniae* ATCC 700603 as a positive control were provided by AHRI and TASH respectively, and used to maintain the quality of laboratory work.

2.9. Data processing and analysis

The collected and laboratory generated data were processed and analyzed using the IBM SPSS version 25.0 computer software and Stata SE 11 software. Rates, types, and resistance pattern of the bacterial isolates with the respective antibiotics was calculated by using Stata SE 11 software. Data was presented in descriptive measures such as a table, figures and percentage. The association between ESBL production and demographic factors was investigated using binary logistic regression model on IBM SPSS version 25.0. Associations were considered statistically significant at a p value of < 0.05.

2.10. Ethical consideration

This study was carried out after it was approved by Addis Ababa University, College of Health Sciences, Department of Microbiology, Immunology and Parasitology Research Ethical Review committee (DRERC) and Armauer Hansen Research Institute (AHRI/ALERT) Ethical Review Committee (AAERC). Support letter was obtained from the study site. The purpose of the study was explained to each participant and isolates were processed only after each participant gives his/her consent or assent. After the completion of tests, the laboratory results were reported to the attending physician for the purpose of patient management.

2.11. Dissemination of results

Upon completion of the study, brief meeting was made with TASH collaborators. And also results were shared with Addis Ababa University and AHRI. It will be also submitted for scientific publication and will be presented in scientific conferences or meetings.

CHAPTER III: RESULTS

3.1 Socio-Demographic characteristics of study participants

A total of 287 study participants who were culture positive for *Enterobacteriaceae* attending different departments of Tikur Anbessa Specialized Hospital from July to September 2018 were included. The median age of the study participants was 9 years with age range of 1 day to 90 years. Fifty (17.4 %) of the participants were neonates, 92(32%) were children under 15 years and 43(14.9%) were patients age greater than 45 years. Majority of the participants (55.7%) were male and the sex ratio male: female was 1.3:1. As presented in the Table 3.1 the majority of the participants were from pediatrics, medical and ICU wards accounting for about 35.9 %, 26.5 % and 26.1%, respectively. From the total study participants, 42 (14.6%) were outpatients and 245 (85.4%) were hospitalized patients.

Out of the 287 study participants about 228(80%) has an exposure to antibiotics in the past three months and the majority of them were exposed to cephalosporin group of antibiotics 152(66.7%) followed by carbapenem 32(14%). About thirty six percent of study participants had a previous hospital admission record but both exposure to antibiotics and hospital admission in the past three month showed no significant association with ESBL production.

Table 3.1:The magnitude of ESBL producing *Enterobacteriaceae* in the world

Sex			Number	Frequency
		Male	160	55.7
		Female	127	44.3
Age	Median 9 years	<28 days	50	17.4
		29 days-1year	20	6.9
		>1-15 years	92	32
	Range 1D-90Y	16-45 years	82	28.6
		>45 years	43	14.9
Patient type		In	245	85.4
		Out	42	14.6
Ward type		Pediatrics	88	35.9
		ICU	64	26.1
		Medical ward	65	26.5
		Surgical ward	6	2.4
		Orthopedics	7	2.9
		Emergency	16	6.5
Exposure to antibiotics		Cephalosporin	152	52.8
		Aminoglycoside	18	6.2
		Quinolones	6	2.0
		Folate pathway inhibitor	7	2.4
		Carbapenems	32	11.1
		Beta-lactam inhibitor	13	4.5
Previous hospital admission		Yes	105	36.6
		No	182	63.4

3.2. Distribution of *Enterobacteriaceae* isolates

Out of all *Enterobacteriaceae* collected, the common isolates were *K. pneumoniae* and *E.coli* accounting for 39% (112/245) and 36.2% (104/245) respectively. The most frequent isolates found among males were *K. pneumoniae* 66 (41.25%) and *E. coli* 51(31.87%) and among females were *E. coli* 53(41.73%) and *K. pneumoniae* 46(36.22%). The dominant bacterium identified from outpatients was *E. coli* (24; 57.1%) while from inpatients was *K. pneumoniae* (100; 89.3%). The majority of isolates in the pediatric ward were also *K.pneumoniae* (66, 58.9%) *and E.coli* (39, 37.5%). *E. coli* was the major isolate from age group greater than 16 years; while *K.pneumoniae* was common in age group younger than 15 years as shown in the Table 3.2. Furthermore, from the other *Enterobacteriaceae* isolates *Citrobacter* spp accounted for 24% (6/25), *Enterobacter* spp, *Providencia* spp, *Salmonella* spp, and *Morganella* spp accounted 16%(4/25) each and the remaining 12% was accounted by *Serratia* spp.

Table 3.2: Distribution of Enterobacteriaceae isolates against socio- demographic characteristics

	***Enterobacteriaceae* isolates, N (%)**						
	Organism	*E.coli* 104(36.2)	*K.pneumoniae* 112(39)	*K. oxytoca* 32(11.1)	*Proteus spp* 14(4.5)	Other-E 25 (8.7)	Total 287(100)
Sex	M	51(49)	66(58.9)	18(56.2)	9(64.3)	16(64)	160(55.7)
	F	53(51)	46(41.1)	14(43.8)	5(35.7)	9(36)	127(44.3)
Age	<28 days	6(5.8)	30(26.8)	9(28)	1(7.1)	4(16)	50(17.4)
	29 days-1year	6(5.8)	7(6.3)	3(9.4)	1(7.1)	3(12)	20(6.9)
	>1-15 years	34(32.7)	40(35.7)	10(31.3)	2(14.3)	6(24)	92(32)
	16-45 years	35(36.7)	24(21.4)	9(28)	6(42.9)	8(32)	82(28.6)
	>45 years	23(22)	11(9.8)	1(3.1)	4(28.6)	4(16)	43(14.9)
Patient type	In	80(76.9)	100(89.3)	28(87.5)	13(93)	24(96)	245(85.37)
	Out	24(23.1)	12(10.7)	4(12.5)	1(7)	1(4)	42(14.63)
Ward type (Inpatient)	Pediatrics	30(37.5)	41(41)	11(39.3)	1(7.7)	5(20.3)	88(35.9)
	Intensive care unit	11(13.7)	36(36)	9(32.1)	2(15.4)	6(25)	64(26.1)
	Medical ward	31(38.8)	18(18)	4(14.3)	4(30.8)	8(33.3)	65(26.5)
	Surgical ward	2(2.5)	2(2)	0( )	1(7.7)	1(4.2)	6(2.4)
	Orthopedics	1(1.3)	2(2)	2(7.1)	1(7.7)	1(4.2)	7(2.9)
	Emergency	5(6.3)	1(1)	2(7.1)	4(30.8)	3(12.5)	16(6.5)

Keys: Other-E = Other *Enterobacteriaceae*: *Enterobacter* spp, *Citrobacter* spp, *M.morgani*, *Providencia* spp, *Serratia* spp *and Salmonella* spp.

Enterobacteriaceae isolates were recovered from patients' clinical specimens including urine (n=104 isolates), blood (n=96 isolates), wound (n=67 isolates), body fluids (n=14 isolates) and other (n=6 isolates). From the total *K. pneumoniae*, 51 (45.5%) followed by 30(26.8%) were from blood and urine respectively. Whereas, 61 (58.6%) followed by 20 (19.2%), 17 (16.3%) of *E. coli* were from urine, wound and blood respectively as presented in Table 3.3.

Table 3.3:Distribution of enteric pathogens isolated from patients clinical specimen at TASH, 2018

Types of enteric pathogens	Clinical Specimens					
	Blood No. (%)	Urine No. (%)	wound No. (%)	Body fluid No. (%)	Other specimen No. (%)	Total
E.coli	17(16.3)	61(58.6)	20(19.2)	6(5.8)	0	104(36.2)
K.pneumoniae	51(45.5)	30(26.8)	21(18.7)	7(6.3)	3(2.7)	112(39)
K.oxytoca	17(53.1)	8(25)	6(18.8)	0	1(3.1)	32(11.1)
Proteus spp	2(14.3)	2(14.3)	9(64.3)	1(7.1)	0	14(4.5)
Other-Enteric bacteria	9(36)	3(12)	11(44)	0	2(8)	25(8.7)
Total	96(33.4)	104(36.2)	67(23.4)	14(4.8)	6(2)	287(100)
Other-Enteric bacteria =*Enterobacter* spp (4), *Citrobacter* spp (6), *M.morgani* (4), *Providencia* spp (4), *Serratia* spp (3) *and Salmonella* spp (4), Other-specimen=stool and sputum						

3.3. General Antimicrobial susceptibility patterns of *Enterobacteriaceae* isolates

Antibiotic susceptibility of the isolates was evaluated against 15 antimicrobial agents by Kirby Bauer disk diffusion method. The overall resistance patterns of the isolates are shown in the Table 3.4. High antibiotic resistance rates were observed to ampicillin 278(96.9%), cefuroxime 259(90.2%), and trimethoprim/sulphamethoxazol 247(86.0%). Among beta lactam/ beta lactam inhibitor combination high level of resistance was observed to amoxicillin/clavulanic acid combination (83.6%). However, amikacin and meropenem had the lowest resistance rates with 9.0 % and 14.6 %, respectively. Species-specific *in-vitro* resistance of the antibiotics revealed that more than 61.5 % of *E.coli* isolates were resistant to *(*cefoxitin, cefotaxime, ceftriaxone, ceftazidime, cefipeme, ciprofloxacillin, trimethoprim/sulphamethoxazol and amoxicillin/

clavulanic acid) and low rate of resistance were observed to amikacin(6.7%) and meropenem (3.8%). On the other hand more than 80 % *K.pneumoniae* isolates were resistant to (cefotaxime, ceftriaxone, ceftazidime, cefipeme, trimethoprim/sulphamethoxazol and amoxicillin/clavulanic acid) and low resistance rate was observed to amikacin (8%). Furthermore, from the other member of *Enterobacteriaceae*, *Citrobacter* spp had lower resistance to pipracillin/tazobactam and amikacin 16.7% (1/6), gentamycin and tobramycin 33.3% (2/6). And also, *Enterobacter* spp showed a resistance rate of 50 % to 100 % to all tested antibiotics other than amikacin (0%). Relatively, *Salmonella* spp have showed low rate of resistance to all tested antibiotics. Generally, *Enterobacteriaceae* isolates in this study had relatively lower resistance rate to aminoglycoside groups, amikacin, gentamicin and tobramycin 9.06 %, 54.01 %, and 37.28 %, respectively.

Table 3.4: Antimicrobial Resistance pattern of Enterobacteriaceae isolates from patients clinical specimens at TASH, 2018

Organism isolated (N)	Antimicrobial resistance level of *Enterobacteriaceae* isolates, N, (%)											
	AM	AMC	PTZ	GN	AK	TOB	CRX	CXT	CTX	CRO	CAZ	FEP
E. coli(104)	100 (96.2)	91 (87.5)	40 (38.5)	42 (40.4)	7 (6.7)	35 (33.6)	93 (89.4)	64 (61.5)	82 (78.8)	83 (79.8)	72 (69)	75 (72)
Enterobacter spp*(4)*	4(100)	4(100.)	2(50)	4(100)	0(0)	2(50)	4(100.)	4(100)	3(75)	3(75)	3(75)	2(50
Citrobacter spp*(6)*	6(100)	4(66.7)	1(16.7)	2(33.3)	2(33.3)	2(33.3)	4(66.7)	3(50)	4(66.7)	4(66.7)	3(50)	3(50)
K.oxytoca(32)	31 (96.8)	22 (68.7)	9 (28.1)	17 (53.1)	4 (12.5)	12 (37.5)	26 (81.3)	15 (46.9)	26 (81.3)	26 (81.3)	18 (56.3)	19 (59.4
K.pneumoniaeae(112)	112 (100)	99 (88.4)	57 (50.9)	78 (69.6)	9 (8)	47 (41.9)	109 (97.3)	66 (58.9)	107 (95.5)	107 (95.5)	91 (81.3)	92 (82.1
M.morgani(4)	4(100)	3(75)	1(25)	2(50)	0(0)	1(25)	3(75)	3(75)	2(50)	2(50)	2(50)	2(50)
Proteus spp*(14)*	13 (92.9)	9 (64.3)	1 (7)	5 (35.7)	2 (14.3)	4 (28.6)	11 (78.6)	9 (64.3)	9 (64.3)	9 (64)	8 (57)	6 (42.8
Providencia spp*(4)*	4(100)	4(100)	2(50)	3(75)	1(25)	2(50)	4(100)	4(100)	4(100)	3(75)	3(75)	4(10
Serratia spp*(3)*	3(100)	3(100)	2(66.7)	2(66.7)	0(0)	2(66.7)	3(100)	2(66.7)	2(66.7)	2(66.7)	2(66.7)	2(66.
Salmonella spp*(4)*	1(25)	1(25)	0(0)	0(0)	1(25)	0(0)	2(50)	1(25)	1(25)	1(25)	1(25)	1(25)
Total(287)	278 (96.8)	240 (83.6)	115 (40.1)	155 (54.)	26 (9.1)	107 (37.3)	259 (90.2)	171 (59.6)	240 (83.6)	240 (83.6)	203 (70.7)	206 (71.8

Keys: AM=ampicillin,GN=gentamicin,AK=Amikacin, CTX=Cefotaxime, CAZ=Ceftazidime, CIP=Ciprofloxacillin, TOB= tobramycin, FEP= cefipeme, CXT=cefoxitin, ME= Meropenem, SXT= trimethoprim/sulphametoxazol, PTZ= pipracillin-tazobactam, AMC= amoxacillin/clavulanic acid and CRX= cefuroxime.

3.4 Magnitude of ESBL producing *Enterobacteriaceae*

From the total 287 *Enterobacteriaceae*, 242(84.3%) isolates showed less than 25mm of inhibition zone to ceftriaxone, 240(83.6%) isolates showed less than 27mm of inhibition zone to cefotaxime and 230(80.1%) isolates showed less than 22mm of inhibition zone to ceftazidime. Therefore, they were subjected to phenotypic confirmation for ESBL activity, of which 74.4% (180/242) isolates and overall 62.7% (180/287) had ESBL activity. From these isolates with ESBL activity 81 isolates (45%) were *K.pneumoniae*, 61 isolates (33.9%) were *E. coli*, 21 isolates (11.7%) were *K.oxytoca*, 7 isolates (3.8%) were *Proteus* spp, 10 isolates (5.6%) were other *Enterobacteriaceae* (Figure 3.2). Among the other ESBL producing *Enterobacteriaceae* isolates 4/10(40%) were *Citrobacter* spp, 3/10(30%) were *Providencia* spp and *Enterobacter* spp, *M. morgani* and *Salmonella* spps were 1/10(10%) each.

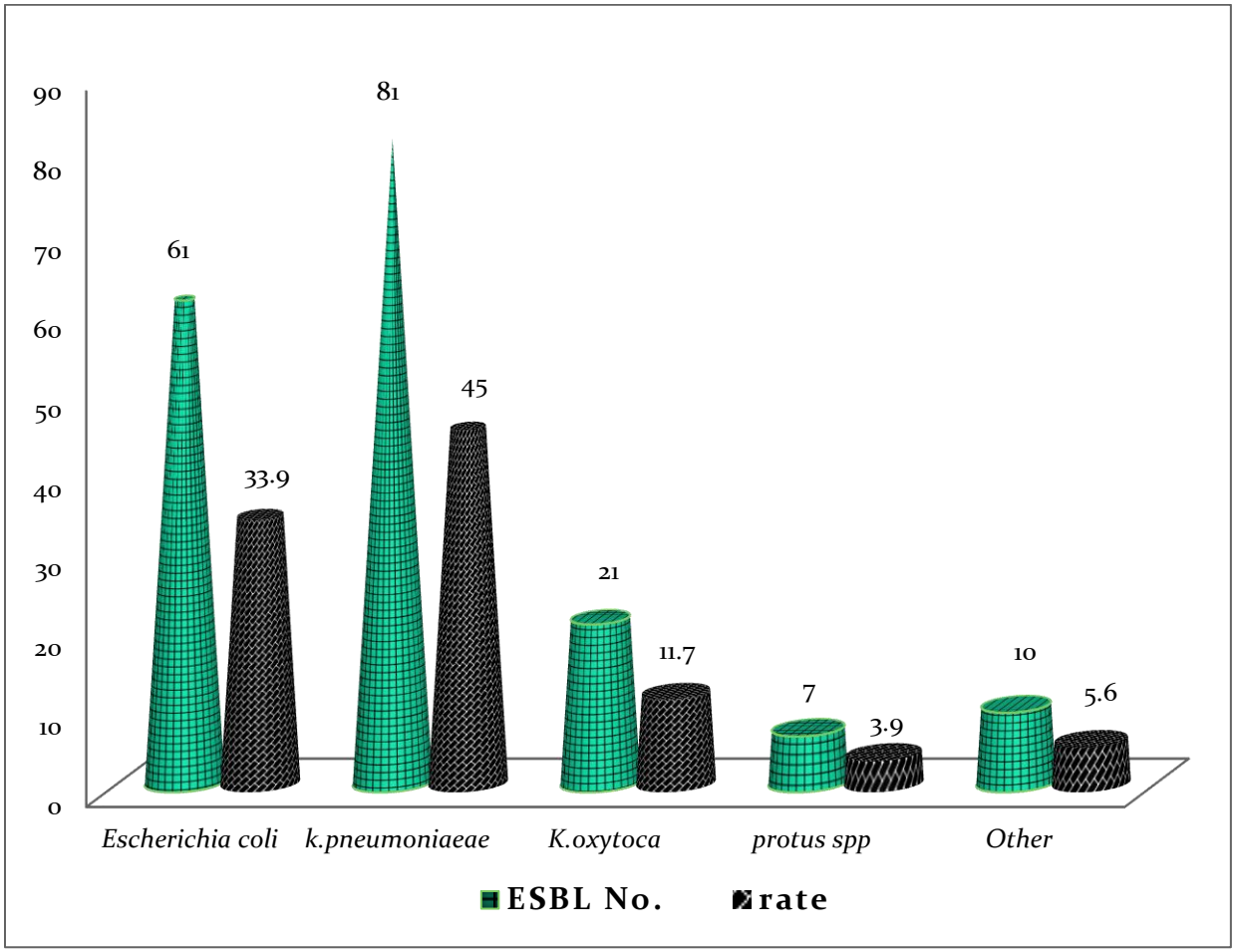

Figure 3.1: Frequency of ESBL producing *Enterobacteriaceae*. Other = *Enterobacter* spp *(1), Citrobacter* spp *(4), M.morgani (1), Providencia* spp *(3), and Salmonella* spp *(1).*

The magnitude of ESBL among hospitalized patients was 64.5% (158/245), which was higher than outpatients 52.3% (22/42) (P>0.05, at 95% CI). Furthermore, ESBL-*Enterobacteriaceae* prevalence was comparably similar between male and female, 53.3% and 46.7%, respectively. In addition, ESBL production was high among isolates from Pediatrics ward, 57/180 (31.7%) followed by Medical ward 42/180(23.3%) as shown in the Table 3.5.

Out of the total 112 *K.pneumoniae* isolates, 81(72.3%) were ESBL producing. Of these isolates 44/81(54.3%) were from male and 37/81(45.7%) were from female. From 81 ESBL positive *K.pneumoniae* isolates 40 (49.4%), 23(28.4%), 13(16 %) and 5(6.2%) were from blood, urine, wound site discharge and body fluid specimens respectively. Among the 81(45%) ESBL producing *K.pneumoniae* isolates 73(90.1%) were from hospitalized patients and 8(9.9%) of them were from outpatients. The magnitude of ESBL producing *K.pneumoniae* were high in the age group younger than fifteen years (59, 72.8%) from this, infants age less than 28 days accounted for 40.7% (24/59). The overall magnitude of ESBL positivity rate among the 104 *E.coli* isolates was 58.7% (61/104). From these 61 ESBL positive *E.coli* isolates, 54.1% (33/61) was from urine and female. Among total isolates of *E.coli* from hospitalized and outpatients 61.2% (49/80) and 50% (12/24) were ESBL producer respectively as indicated in Table 3.5.

Table 3.5:Distribution of ESBL producing Enterobacteriaceae isolates in relation to socio-demographic characteristics N (%)

Variables		*E. coli (61)*	*K.pneumoniae* (81)	*K.oxytoca*(21)	*Proteus* spp(7)	Other-*E* (10)	Total (180)
Sex	Male	28(45.9)	44(54.3)	13(61.9)	4(57.1)	7(70)	96(53.3)
	Female	33(54.1)	37(45.7)	8(38.1)	3(42.9)	3(30)	84(46.7)
Age	<28 day	5(8.2)	24(29.6)	8(38.1)	0	1(10)	38(21.1)
	29 day-1year	3(4.9)	5(6.2)	0	0( )	1(10)	9(5)
	>1-15 year	18(29.5)	30(37)	6(28.6)	1(14.3)	3(30)	58(32.2)
	16-45 year	20(32.8)	16(19.7)	6(28.6)	5(71.4)	2(20)	49(27.2)
	>45 year	15(24.6)	6(7.4)	1(4.7)	1(14.3)	3(30)	26(14.4)
Patient settings	In	49(80.3)	73(90.1)	19(90.4)	7(100)	10(100)	158(87.8)
	Out	12(19.7)	8(9.9)	2(9.6)	0	0	22(12.2)
Samples type	Blood	13(21.3)	40(49.4)	11(52.4)	1(14.3)	2(20)	67(37.2)
	Urine	33(54.1)	23(28.4)	6(28.6)	1(14.3)	2(20)	65(36.1)
	Wound	10(16.4)	13(16)	3(14.3)	5(71.4)	5(50)	36(2)
	Body fluid	5(8.2)	5(6.2)	0	0	0	10(5.6)
	Other specimen	0	0	1(4.7)	0	1(10)	2(1.1)
Ward type	Pediatrics	17(27.9)	31(38.3)	7(33.3)	0	2(20)	57(31.7)
	Intensive care unit	6(9.8)	25(30.8)	6(28.6)	2(28.7)	2(20)	41(22.8)
	Medical ward	21(34.4)	13(16)	3(14.3)	2(28.7)	3(30)	42(23.3)
	Surgical ward	1(1.6)	2(2.5)	0()	1(14.3)	1(10)	5(2.8)
	Orthopedics	0	2(2.5)	2(9.5)	0	1(10)	5(2.8)
	Emergency	4(6.6)	1(1.2)	1(4.7)	2(28.7)	1(10)	9(5)
Total	180 (62.7)	61(33.9)	81(45.0)	21(11.7)	7(3.8)	10(5.6)	

Keys: Other *Enterobacteriaceae*= *Enterobacter* spp *(1)*, *Citrobacter* spp *(4)*, *M.morgani (1)*, *Providencia* spp *(3), and Salmonella* spp *(1).*

3.5. Association between ESBL production and socio-demographic factors

In our study isolates from female patients had higher proportion of ESBL positivity (66.1%) than those from male patients (60%). And also, Patients with age range 1-15 years had shown greater positivity (63%), as compared with other age groups but no significant association was observed (*P*=0.66, OR=1.15 at 95% CI). The highest intra-species frequency of ESBL production was observed among *K.pneumoniae* 72.3% (81/112) followed by *K.oxytoca* 65.6% (21/32) and *E.coli* 58.7% (61/104). Logistic regression analysis indicated that *K.pneumoniae* was significantly responsible to develop ESBL than other *Enterobacteriaceae* isolates (*P*=0.003, Odds ratio=3.919 (1.592-9.649) at 95% CI). Even though, *Enterobacteriaceae* isolates from surgical ward showed highest ESBL positivity of 83.3%, no significant association was observed between ESBL-producing *Enterobacteriaceae* and ward types. The lowest rate of ESBL positivity was observed

in Emergency wards (56.3%) for hospitalized patients. Higher rate of ESBL production was observed in blood and urine samples, 69.8% and 62.5 % respectively as shown in Table 3.6.

Table 3.6:Association between ESBL production and socio-demographic characteristics

Variables (Numbers)		ESBL positive N (%)	ESBL negative N (%)	OR	P value
Sex	Male (160)	96(60)	64(40)	0.768	0.286
	Female (127)	84(66.1)	43(33.9)		R
Age	<28 day	38(76)	12(24)	2.13	0.06
	29 day-1year	9(45)	11(55)	0.23	0.55
	1-15 year	58(63)	34(37)	1.15	0.66
	16-45 year	49(59.7)	33(40.3)		R
	>45 year	26(60.4)	17(39.6)	1.03	0.94
Ward type	Pediatrics (88)	57(64.8)	31(35.2)	1.007	0.984
	Intensive care unit (64)	41(64)	23(36)	0.976	0.948
	Medical ward (65)	42(64.6)	23(35.4)		R
	Surgical ward (6)	5(83.3)	1(16.7)	2.738	0.371
	Orthopedics (7)	5(71.4)	2(28.6)	1.369	0.720
	Emergency (16)	9(56.3)	7(43.7)	0.704	0.536
Organisms	*E.coli* (104)	61(58.7)	43(41.3)	2.128	0.096
	K.pneumoniae (112)	81(72.3)	31(27.7)	3.919 (1.592-9.649)	0.003
	K.oxytoca (32)	21(65.6)	11(34.4)	2.864	0.057
	Proteus spp (14)	7(50)	7(50)	1.500	0.547
	Other-E (25)	10(40)	15(60)		R
Samples	Blood (96)	67(69.8)	29(30.2)	4.621	0.087
	Urine (104)	65(62.5)	39(37.5)	3.333	0.176
	Wound site discharge (67)	36(53.7)	31(46.3)	2.323	0.349
	Body fluid (14)	10(71.4)	4(18.6)	5.000	0.125
	Other specimen (6)	2(33.3)	4(66.7)		R

Keys: Other-E *E*: *Enterobac* spp, *Citrobacter* spp, *M.morgani, Providencia* spp, *Serratia* spp *and Salmonella* spp. Other specimens: stool and sputum. R= reference

3.6. Comparison of Antimicrobial susceptibility patterns between ESBL and non-ESBL *Enterobacteriaceae* isolates

Table 3.7 shows the antibiotics resistance profile of ESBL producing and none producing isolates against commonly prescribed antibiotics. All ESBL producing isolates were 100% resistant to ampicillin, 98.9% to cefotaxime, 98.3% to ceftriaxone, 87.8% to amoxicillin/clavulanic acid, 82.2% to cefipeme, 78.9% to ceftazidime, 61.1% to ciprofloxacillin and 60.6% to gentamicin. On the one hand non ESBL isolates were 91.6% resistant to ampicillin, 76.6% to amoxicillin/clavulanic acid, 58.9% to ceftriaxone, 57.9% to cefotaxime, 57% to ceftazidime, 54.2% to ciprofloxacillin, 54.2% to cefipeme and 43% to gentamicin. The ESBL-producing isolates (n = 180) significantly ($P < 0.05$) had increased resistance compared with

non–ESBL producers (n = 127) to cotrimoxazole (92.8% vs 74.7%), ciprofloxacin (61.1% vs 54.2%) and gentamicin (60.6% vs 43%). Despite higher antibiotics resistance above, non ESBL isolates were highly resistant to amikacin (11.2 % vs 7.2 %) and meropenem (29 % vs 5.5%) antibiotics than ESBL producers respectively.

Table 3.7: Antimicrobial resistance patterns of confirmed ESBL producing and non-producing *Enterobacteriaceae*

Groups of Antibiotic	Antibiotics	ESBLs (180)	Non ESBLs (107)	P value
		N (%)	N (%)	
Penecillins	AM (10 µg)	180(100)	98(91.6)	<0.05
2nd GC	CRX(30 µg)	179(99.4)	80(74.8)	<0.05
3rd GC	CTX(30 µg)	178(98.9)	62(57.9)	<0.05
	CRO(30 µg)	177(98.3)	63(58.9)	<0.05
	CAZ (30 µg)	142(78.9)	61(57)	<0.05
4th GC	FEP(30 µg)	148(82.2)	58(54.2)	<0.05
Aminoglycosides	GN(10 gµ)	109(60.6)	46(43)	<0.05
	AK(30 gµ)	13(7.2)	12(11.2)	0.321
	TOB(30 µg)	68(37.8)	39(36.4)	0.419
Fluoroquinolones	CIP(5 gµ)	110(61.1)	58(54.2)	<0.05
Cephamycins	CXT(30 µg)	92(51.1)	79(73.8)	<0.05
Carbapenem	ME (10 µg)	10(5.5)	31(29)	<0.05
Folate pathway inhibitors	SXT(25 µg)	167(92.8)	80(74.7)	<0.05
Penecillins + Beta--lactamase inhibitors	PTZ(100/10 µg)	58(54.2)	57(53.3)	0.311
	AMC((20/10 µg)	158(87.8)	82(76.6)	<0.05

Keys: AM=ampicillin, GN=gentamicin, AK=Amikacin, CTX=Cefotaxime, CAZ=Ceftazidime, CIP=Ciprofloxacillin, TOB= tobramycin, FEP= cefipeme, CXT= cefoxitin, ME= Meropenem, SXT= trimethoprim/sulphamethoxazol, PTZ= pipracillin-tazobactam, AMC= amoxacillin/clavulanic acid and CRX= cefuroxime.

Table 3.8 shows resistance patterns of ESBL producing species of *Enterobacteriaceae* isolates in relation with none producers. ESBL producing *E.coli* was found to be more resistant to (ceftriaxone and cefuroxime) 100%, to ceftazidime 83 %, to ciprofloxacillin 77%, to cefipeme 86.9% and amoxacillin/clavulanic acid 91.8% than ESBL producing *K.pneumoniae* which was resistant to (ceftriaxone and cefuroxime) 98.7%, to ceftazidime 79 %, to ciprofloxacillin 49.4%, to cefipeme 82.7 % and to amoxacillin/clavulanic acid 87.6%. However, ESBL producing *K.pneumoniae* isolates were highly resistant to amikacin (4.9% vs 3.3%) and meropenem (7.4% vs 0%) than ESBL producing *E.coli* isolates. From ESBL producing *Enterobacteriaceae* isolates, high level of resistance to amikacin was observed in ESBL-*Proteus* spp (28.6%) and

low level of resistance (3.3%) was observed in ESBL-*E.coli*. ESBL producing *K.oxytoca* and *Proteus* spp had showed a similar higher resistance to meropenem (14.3%). No resistance to meropenem was observed among ESBL-*E.coli* isolates. ESBL negative *K.pneumoniae* isolate was resistant to amikacin 16.1% and to meropenem 58 % which is greater than ESBL positive *K.pneumoniae* 4.9% to amikacin and 7.4 % to meropenem.

Table 3.8: Comparisons of AST patterns between the different species of ESBL producing and none producing Enterobacteriaceae

ESBL pattern	Resistance patterns %														
	AMP	GEN	AMK	CTX	CRO	CAZ	CIP	TOB	FEP	CXT	MEM	SXT	PTZ	AMC	CRX
ESBL-EC^{+}(61)	100	49.1	3.3	98.4	100	83.6	77	32.8	86.9	52.5	0	90.2	31.1	91.8	100
ESBL-EC^{-}(43)	90.7	27.9	9.3	51.1	48.8	48.8	55.8	34.9	51.1	74.4	6.9	83.7	48.8	81.4	74.4
ESBL-K.P $^{+}$(81)	100	69.1	4.9	98.7	98.7	79	49.4	39.5	82.7	48.1	7.4	95	38.3	87.6	98.7
ESBL-K.P $^{-}$(31)	100	70.9	16.1	87	87	87	67.7	48.4	80.6	87	58	87	83.7	90.3	93.5
ESBL-K.O $^{+}$(21)	100	61.9	14.3	100	95.2	61.9	47.6	38.1	71.4	38.1	14.3	95.2	23.8	71.4	100
ESBL-K.O $^{-}$(11)	90.9	3.4	9.1	45.5	54.5	45.5	54.5	36.4	36.4	63.6	27.3	72.7	36.4	63.6	45.5
ESBL -Pr^{+}(7)	100	57.1	28.6	100	85.7	85.7	71.4	42.8	57.1	71.4	14.3	100	14.3	85.7	100
ESBL-Pr^{-}(7)	85.7	14.3	0	28.6	28.6	28.6	14.3	14.3	28.6	57.1	14.3	42.8	0	42.8	57.1
ESBL-Oth^{+} (10)	100	60	20	100	90	80	80	5	90	80	0	80	20	100	100
ESBL- Oth $^{-}$ (15)	80	46.7	13.3	40	40	40	40	26.7	33.3	60	40	40	40	60	66.7

Keys: AMP=Ampicillin, GEN=Gentamycin, AMK=Amikacin, CRX=Cefuroxime, CTX=Cefotaxime, CRO= Ceftriaxone, CAZ= Ceftazidime, FEP=Cefepime, TOB=Tobramycin, CIP= Ciprofloxacin , CXT = Cefoxitin, MEM=Meropenem, SXT= Trimethoprim-sulfamethoxazole, AMC= Amoxicillin-clavulanic acid and PTZ= Pipracillin-tazobactam : ESBL-EC^{+}=ESBL positive *E.coli*, ESBL-EC^{-} ESBL negative *E.coli*, ESBL-K.P $^{+}$= ESBL positive *K.pneumoniae*, ESBL-K.P $^{-}$ = ESBL negative *K.pneumoniae*, ESBL-KO^{+}= ESBL positive *K.oxytoca*, ESBL-KO^{-} ESBL negative *K.oxytoca*, ESBL- Pr^{+} ESBL positive *Proteus* spp, ESBL -Pr $^{-}$ ESBL negative *Proteus* spp, ESBL-Oth^{+}= ESBL positive other *Enterobacteriaceae*, ESBL-Oth^{--} = ESBL negative other *Enterobacteriaceae*

3.7. Magnitude of MDR isolates

Resistance to three or more class of antimicrobials was observed in 91.9% (264) isolates, among which *K.pneumoniae* and *E.coli* contributed to 41.5% (110/264) and 36.2 % (96/264) respectively. Among the MDR isolates (non-susceptible to at least 3 antibiotics belonging to different antibiotics categories) only 8.3% (22) isolates were resistant to at least one agent in three classes of antibiotics. Furthermore, 12.9% (34/264), 17.8% (47/264), 25.8% (68/264), 25.3% (67/264), 9.8% (26/264) of *Enterobacteriaceae* isolates were found to resistant to four, five, six, seven and eight class of antibiotics respectively as shown in Table 3.9.

Magnitude of drug resistance pattern compared within species showed that, 98.2% *K.pneumoniae* and 92.3% *E.coli* were MDR. From all MDR isolates of *E. coli* 25/96(26%) showed resistance to all class of antibiotics with the exception to carbapenem. Among ESBL negative *Enterobacteriaceae* isolates, 96.7% *K.pneumoniae* were MDR which was the highest (Table 3.9). Out of all *Enterobacteriaceae* isolates 26/264(9.8%) were resistant to all tested antibiotics. Of these isolates 19.3% were ESBL producers, the remaining 80.7% were ESBL non-producers. Higher rate of MDR pattern was observed in *Enterobacteriaceae* isolates that originated from hospitalized patients (93%) than *Enterobacteriaceae* isolates from the out patients, (85.7%).

Table 3.9: Multidrug resistance level of *Enterobacteriaceae* to different classes of antibiotics in TASH between July to September 2018

Isolates(N)	**Level of drug resistance ((N (%))**									
	R0	R1	R2	R3	R4	R5	R6	R7	R8	Total MDR(>R3)
E.coli	3 (2.8)	2 (1.9)	3 (2.8)	8 (7.7)	15 (14.4)	12 (11.5)	35 (33.6)	25 (24)	1 (0.9)	96 (92.3)
Enterobacter spp	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25)	0 (0.0)	0 (0.0)	1 (25)	2 (50)	4 (100)
Citrobacter spp	0 (0.0)	2 (33.)	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	4 (66.6)
K.oxytoca	0 (0.0)	2 (6.3)	2 (6.9)	3 (10.3)	5 (17.2)	5 (15.6)	5 (17.2)	7 (21.9)	3 (10.3)	28 (87.5)
K.pneumoniaeae	0 (0.0)	1 (0.9)	1 (0.9)	6 (5.4)	9 (8)	29 (25.9)	26 (23.2)	23 (20.5)	17 (15.2)	110 (98.2)
M.morgani	0 (0.0)	0 (0.0)	0 (0.0)	2 (50)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25)	1 (25)	4 (100)
Proteus spp	0 (0.0)	3 (21.4)	1 (7.1)	2 (14.3)	1 (7.1)	0 (0.0)	1 (7.14)	5 (35.7)	1 (7.14)	10 (71.4)
Providencia spp	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25)	0 (0.0)	2 (50)	1 (25)	4 (100)
Serratia spp	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	1 (33.3)	1 (33.3)	0 (0.0)	3 (100)
Salmonella spp	2 (50)	1 (25)	0(0.0)	0 (0.0)	1 (25)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25)
Total (287)	5 (1.9)	11 (4.2)	7 (2.7)	22 (8.3)	34 (12.9)	47 (17.8)	68 (25.8)	67 (25.3)	26 (9.8)	264 (91.9)

R0= no resistance, R1= resistant to one class of antibiotics, R2=resistant to two class of antibiotics, R3=resistant to three class of antibiotics, R4=resistant to four class of antibiotics, R5= resistant to five class of antibiotics, R6=resistant to six class of antibiotics, R7=resistant to seven class of antibiotics, R8=resistant to eight class of antibiotics.

Moreover, More than 98.9% ESBL producing *Enterobacteriaceae* isolates demonstrated MDR pattern which was higher compared with none ESBL *Enterobacteriaceae* isolates (80.4%). ESBL producing isolates were found significantly multi- drug resistant than none producers ($p< 0.05$, OR=0.049(0.011-0.214), at CI 95%). ESBL producing *K.oxytoca, Proteus spp* and ESBL+ other *Enterobacteriaceae* were found 100% MDR as shown in Figure 3.2.

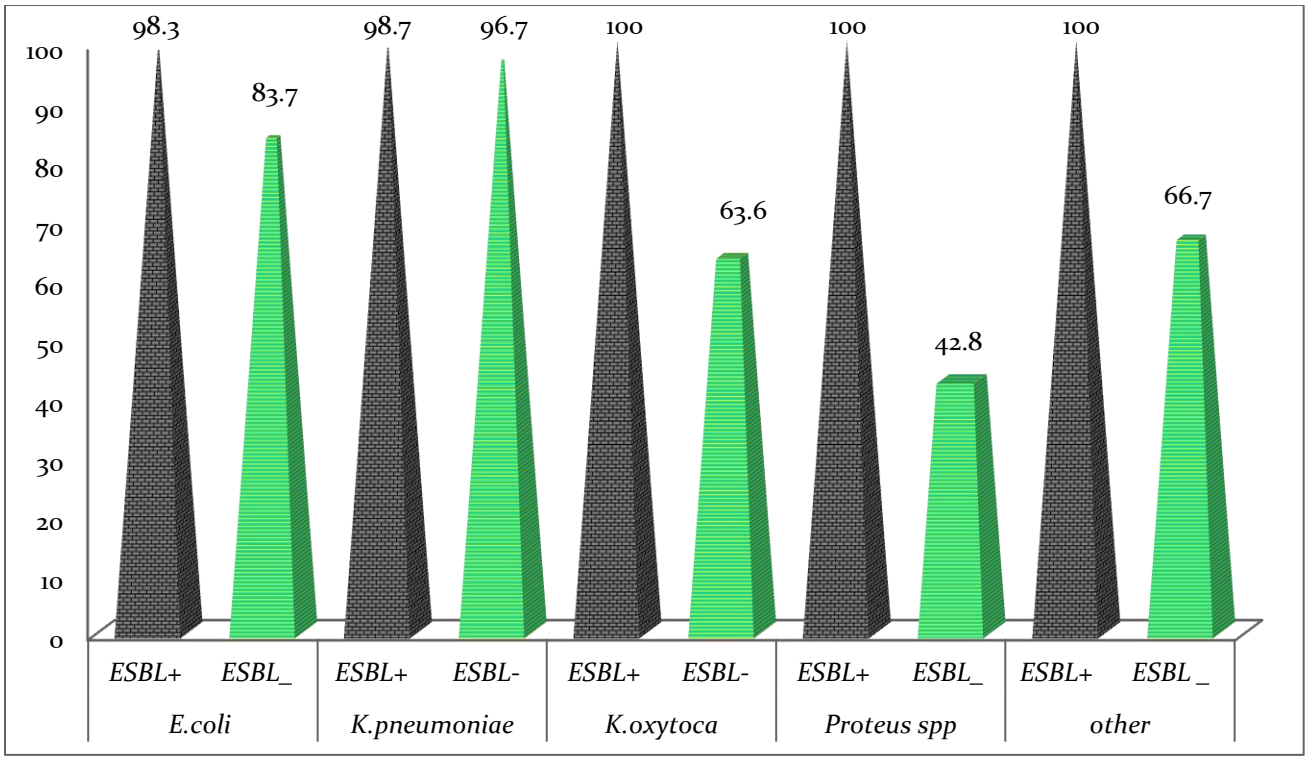

Figure3.2: Magnitude of MDR pattern of ESBL producing and non-producing *Enterobacteriaceae* isolates from patients attending TASH.

CHAPTER IV: DISCUSSION

4.1. Discussion

ESBL producing *Enterobacteriaceae* have become a widespread serious problem. These enzymes are becoming increasingly expressed by many members of *Enterobacteriaceae* family; *Enterobacter* spp, *Citrobacter* spp, *M. morgani*, *Providencia* spp, *Proteus* spp and *Salmonella* spp in this study were found to be ESBL producers, in addition to *E.coli* and *Klebsiella spp*. More than 50% of *Enterobacteriaceae* isolates from in and outpatients, all ward types and all sample types were found ESBL producer. In the present study, *Enterobacteriaceae* species were frequently isolated from children less than fifteen years. Pediatrics ward, Medical wards and ICU wards were the major areas where significant amount of drug resistant and ESBL producing *Enterobacteriaceae* isolates were collected from hospitalized patients. Therefore, recent data about Antimicrobial activity and ESBL pattern is very essential for proper treatment of infections caused by *Enterobacteriaceae* and controlling the spreading of drug resistant species in hospital environments and at the community. Our study demonstrated the magnitude of ESBL producing multidrug resistant *Enterobacteriaceae* and their Antimicrobial activity simultaneously.

4.2. Prevalence of ESBL producing *Enterobacteriaceae*

In the present study, magnitude of ESBL production was 62.7 % which was lower than previous studies in Ethiopia 78.57% (Legese *et al*., 2017), in Uganda 89 % (Andrew *et al*., 2017) and higher than reports from other studies in Ethiopia; 38.4% in Jimma (Siraj *et al*., 2015) and 25% in Adama (Mulisa *et al*., 2016). The reason for the variation could be due to the difference in number of isolates studied, methodology applied and study setting most importantly the clinical settings of the study sites TASH is a tertiary level hospital. Our finding was comparable with other reports in Ethiopia, 63.4% (Zeynudin *et al*., 2018) and India 69% (Umadevi *et al*., 2011) and 54.3%(Vinodhini *et al*., 2014). Magnitude of 62.7 % ESBL production in this study was much higher than reports from Brazil 21.3 % (Nogueira Kda *et al*., 2015), Iran 30.5 % (Moosavian and Deiham 2012), and Taiwan 20.6% (Liu *et al*., 2016). Among the possible cases for this high figure, uncontrolled use of third generation cephalosporin create a selective pressure for emergence of resistant strains due to easy accessibility of the drug in our country, variation in application of proper infection prevention measure which may have a factor in regulating the transmission of ESBL genes in the hospital and community and absences of policies which regulate access to antibiotics in the community. High rate of GI colonization by ESBL producing *Enterobacteriaceae* in Ethiopia could be additional factor for the variation (Desta *et al*., 2016)

K.pneumoniae and *E.coli* were the major contributor 45 % and 33.9% respectively, which is in line with a study done in Ethiopia (Teklu *et al*., 2019) and in India **(**Siddiqui *et al*., 2014). Among *K.pneumoniae* isolates, 72.3 % were ESBL producer while *E.coli* showed 58.7 % which was supported by study in Sudan (Ahmed *et al*., 2013), Iran **(**Oduro-Mensah *et al*., 2016), Ghana (Moosavian and Deiham, 2012), Ethiopia (Abera *et al*., 2016) and (Desta *et al*., 2016). From all isolates of *Enterobacteriaceae K.pneumoniae* was observed to be the significant ESBL producer. This could be due to their propensity to acquire antimicrobial resistance genes (Khaertynov *et al*., 2018).

ESBL producing *K.pneumoniae* was considered mainly a nosocomial pathogen, while ESBL-producing *E.coli* is responsible for hospital and community acquired infections (Ouedraogo *et al*., 2016). In agreement, we identified ESBL producing *K.pneumoniae* most frequently from hospitalized patients. ESBL- E. coli showed no significant difference between hospitalized 62.3% and out patients 50% (p=0.328 OR=0.633 at 95% CI). In our study no significant association was observed on magnitude of ESBL production between hospitalized and outpatients (P=0.136, OR=0.606, 95 % CI) similar report was made by other group (Ogefere *et al*., 2015) in southern Nigeria. The reason could be that, these ESBL producing isolates are spreading in the community besides the hospital environment. More ESBL-producing *Enterobacteriaceae* were isolated from male patients compared to female. However, no significant association of ESBL production with sex (p=0.2860, OR=0.768, 95% CI). But in Burkina Faso male patients had higher production of ESBL (Ouedraogo *et al*., 2016).

Besides, there was no significant difference in the magnitude of ESBL production between *Enterobacteriaceae* isolates from blood, urine, wound site discharge, body fluids and other specimens. The majority of (60-72%) *Enterobacteriaceae* isolates from blood, urine and body fluid were ESBL producer. Therefore, this ESBL producing strains are posing series challenge for diagnosis because the treatment options are limited and in most case they are also multi-resistant. *E. coli* one of the predominate pathogens responsible for urinary tract infection in women was the major isolates from urine and of these 54.1% of them were ESBL producers and this finding was in agreement with a similar study done in Nepal where 72.3 of the *E. coli* from women were ESBL producers. This could be due to that *E.coli* is the major gram negative bacteria causing UTI and its resistance profile is increasing over years (Foxman, 2014)

Klebisella spp was the major isolates from hospitalized patient with highest rate of ESBL production 71.9%. We know from the literature that ESBL producing *Klebisella spp* are associated with nosocomial infections (Ouedraogo *et al*., 2016; Khaertynov *et al*., 2018). to this end the large majority of ESBL producing *Klebisella spp* were from patients admitted in the hospital for one and another reason Therefore, it is important to give special attention to hospitalized patients prior to empirical therapy with antibiotics. However, a study from Sri Lanka reported only 13.8% ESBL producing *Klebisella spp* (Fernando *et al*., 2017).

In our study *K.pneumoniae* isolates from children under the age of fifteen had higher proportion of ESBL positivity (>72.8%) than isolates from adult patients which was supported by study in Ethiopia (Desta *et al*., 2016). Also, we found that *K.pneumoniae* isolated from blood samples had higher positivity of ESBL production (78%). Therefore, rapid detection of ESBL producing *K. pneumonia* in clinical laboratory is essential for timely diagnosis of this resistant organism and management of infection with this pathogen. This was supported by study done by (Ouedraogo et al., 2016). ESBL-*Enterobacteriaceae* isolates from different wards among hospitalized patients were found to be more frequent ESBL producers. However, no significant association was observed being in different wards and colonized by ESBL producing *Enterobacteriaceae*.

4.3. Antibiotics Resistance pattern of *Enterobacteriaceae*

The rate of antimicrobial resistance among all isolates of *Enterobacteriaceae* ranged between 9.1% and 96.8% for amikacin and ampicillin respectively. Resistance to meropenem was found in 14.6% of *Enterobacteriaceae* isolates which was quite higher than for amikacin. ESBL production has enhanced for co-resistance to different classes of antibiotics, trimethoprim/sulphamethoxazol 92.8%, amxacillin/clavulanic acid 87.8%, cefipeme 82.2%, ciprofloxacillin 61.1% and gentamicin 60.6%, as compared with none production as none producers have less resistance to, amxacillin/clavulanic acid 76.6%, trimethoprim/sulphametoxazol 74.7%, cefipeme 54.2%, ciprofloxacillin 54.2%, and gentamicin 43% ($p<0.05$). This result was comparable with study in Ethiopia, amxacillin/clavulanic acid (91.5% vs 0%), trimethoprim/sulphamethoxazol (90.8% vs 84.2%) and cefipeme (97.6% vs 89.5%) by (Teklu *et al*., 2019), in Burkina Faso trimethoprim/sulphamethoxazol (45% vs 5%), ciprofloxacillin (80% vs 12%) and gentamicin(89% vs 27.5%) by (Ouedraogo *et al*., 2016) , in Saudi Arabia,

trimethoprim/sulphamethoxazol (87.1% vs 45.7%), ciprofloxacillin (86.3% vs 36.6%) and gentamicin (59% vs 12.5%) by (Hassan and Abdalhamid 2014) and India, trimethoprim/sulphamethoxazol (82.1% vs 56.1%), ciprofloxacillin (89.9% vs 41.7%), and gentamicin (88% vs13.6%) (Kaur *et al*., 2013). This may be due to the fact that, ESBLs are often encoded by genes located on plasmids, and these also carry genes for resistance to other antimicrobial agents such as aminoglycosides, trimethoprim, sulphonamides, and fluoroquinolones (Fernando *et al*., 2017). One study had demonstrated fluoroquinolones resistance mediated by co-transfer of the *qnr* determinant on ESBL-producing plasmids (Rawat and Nair, 2010).

ESBL producers were least resistant to amikacin 7.2% and meropenem 5.5% which was in close agreement with studies in Ethiopia which have reported resistance of 3.3 % to meropenem and 17.9 % to amikacin in Addis Ababa (Teklu *et al*., 2019), in Jimma 23.5% to amikacin (Abayneh *et al*., 2018), and in other African countries 3.3 % to amikacin in Togo (Salah *et al*., 2019). This could be due to that amikacin was not used as a treatment option for diagnosis of infections caused by this group of bacteria and meropenem was a new drug with little exposure to this group of organism, in this case minimum or little selective pressure was experienced by these organisms to give a response. ESBL producing *K.pneumoniae* in particular was found highly resistant to ampicillin 100%, (cefotaxime, ceftriaxone & cefuroxime) 98.7%, trimethoprim/sulphamethoxazol 95%, amoxicillin/clavulanic acid 87.6% and cefipeme 82.7% and less resistant to amikacin 4.9%, meropenem 7.4% and pipracillin-tazobactam 38.3%. This was in agreement with study in Ethiopia (Desta *et al*., 2016) and (Teklu *et al*., 2019), in Togo (Salah et al., 2019), and in Peru (Garcia *et al*., 2016). The corresponding figure for ESBL *E.coli* was 100% to (ampicillin, ceftriaxone & cefuroxime), 98.4 % to cefotaxime, 91.8 to amoxicillin/clavulanic acid, 90.2% to trimethoprim/sulphamethoxazol and 86.9% to cefipeme and less resistance was observed to meropenem 0%, amikacin 3.3%, pipracillin-tazobactam 31.1% which was in line with study in Sudan (Ibrahim *et al*., 2013) in India (Singh *et al*., 2016). The possible reasons could be expression of co-resistance determining genes besides ESBL encoding genes.

4.4. Multi-drug resistant *Enterobacteriaceae*

In the present study the overall magnitude of MDR was 91.98%. Which was higher than a study done in Ethiopia (68.3%) by (Teklu *et al*., 2019), Jimma Ethiopia (30.2%) by (Gashaw *et al*., 2018). The variation could be that patients are referred to TASH after exposure to different class of antibiotic drugs and the majority of our isolates are from inpatients where different class of antibiotics are switched frequently without laboratory testing. Both are ways of applying selective pressure on bacteria which can aggravate for the emergence of multiple resistant strains. However, 91.9% was fairly comparable with study done in Ethiopia by (Eshetie *et al*., 2015) (86.9%). The present study showed that, *K.pneumoniae* (98.2%) and *E.coli* (92.3%) were the major MDR isolates among the *Enterobacteriaceae* which was similar with study done in Nepal *E.coli* (91.66%) *and K.pneumoniae* (87.5%) by (Chande and Shrestha, 2013) and in Iraq *K.pneumoniae* (75%) and *E.coli* (87.5%) by (Pishtiwan and Khadija, 2019).

The magnitude of MDR in ESBL producing isolates was much higher than none producers (98.9% vs 80.4%). Similarly, higher rate was reported in Thailand (Kiddee *et al*., 2019). ESBL production is significantly associated with MDR patterns $p < 0.05$; for instance, ESBL producing *Proteus* spp had showed 100% MDR pattern whereas ESBL negative *Proteus* spp had showed 42.8%. ESBL producing and none-producing *K.pneumoniae* isolates showed nearly similar MDR patterns. This could be that none ESBL isolates of *K.pneumoniae* harbor carbapenemase enzyme which is responsible to wide range of action because most of ESBL non-producing *K. pneumoniae* isolates were resistant to meropenem. ESBL producing K. oxytoca and Proteus spp were found 100% MDR. From all Enterobacteriaceae isolates 9.8 % were resistant to all antibiotics tested. Among the possible explanation in that ESBLs are often encoded by genes located on plasmids, and these also carry genes for resistance to other antimicrobial agents (Thenmozhi *et al.*, 2014).

Strength and weakness of the study

Strength of the study

- This study included a large number of isolates causing different site infections.
- This study also showed the possible alternative treatments for ESBL producing *Enterobacteriaceae* by doing AST for 15 antibiotic including beta lactam and other groups.
- We have also kept our isolates at -80^{o} C for further molecular characterization of resistance determining genes and virulence gene analysis

Limitation of the study

- We are unable to determine the level of co-expression of ESBL and AmpC due to unavailability of confirmatory tests for AmpC.
- We are also unable to determine extended drug resistant and pan drug resistant isolates due to unavailability of recently used optional drugs like fosfomycin and polymixin B.

Conclusion and recommendation

Conclusion

Enterobacteriaceae isolates exhibited high level of resistance to most of antibiotics tested. Better susceptibility was observed to amikacin followed by meropenem and tobramycin. The magnitude of ESBL production among *Enterobacteriaceae* isolates was found high. Furthermore, highest production of ESBL was observed for *K. pneumoniae* followed by *E. coli, K. oxytoca* and *Proteus spp.* Additionally, from the other *Enterobacteriaceae* isolates two third of *Citrobacter* spp and three fourth of *Providencia* spp were ESBL producers. ESBL producing *Enterobacteriaceae* isolates showed higher resistance to third generation cephalosporins,

gentamicin and amoxicillin/clavulanic acid than non-producers. ESBL non-producing *Enterobacteriaceae* isolates showed higher resistance to meropenem than ESBL producers. Amikiacin was found the most effective drugs for both ESBL producers and non-producers. The magnitude of MDR pattern was alarming among all *Enterobacteriaceae* isolates in this study. Greater figure of MDR pattern was observed for ESBL producers. Higher proportion of ESBL production was observed in participants from age less than 15 to those above 16 years, pediatrics ward was the major area where highest proportion of ESBL producing *Enterobacteriaceae* isolates were observed.

Recommendations

Based on the findings of our study the following recommendations are made:

- Routine screening of patients for ESBL is recommended for controlling the spread of this treat among patients in hospital environment, for applying stringent infection prevention measure and for better management of patients.
- Strong antibiotics ordering policies and utilization policies are required
- Managers of the hospital and the respective stakeholders should work hard to solve this emerging multidrug resistance in *Enterobacteriaceae*
- Further study with large sample size including consequences of being infected by ESBL producing multidrug resistant *Enterobacteriaceae* should be done
- Molecular characterization of ESBL genes should be done to know the responsible ESBL types

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6. Annexes

Annex IA: **Information Sheet for Study Participants**

A. Information Sheet in English

Introduction

My name is Aminu Seman. I am MSc student of Medical microbiology at Department of Microbial, Immunology and Parasitology, College of health science school of medicine, Addis Ababa University (AAU). I am here to conduct study for my MSc thesis on: determining the magnitude of ESBL producing *Enterobacteriaceae* and molecular characterization of ESBL genes.

Title of the research: Phenotypic detection and Molecular characterization of extended-spectrum β-lactamases genes from clinical isolates of *Enterobacteriaceae* in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia

Objective of the study: The aim of this study is to assess the current magnitude of ESBL producing Enterobacteriaceae infections and molecular characterization of ESBL genes in Tikur Anbessa Specialized Hospital Addis Ababa, Ethiopia.

Source of fund: Addis Ababa University and Armauer Hansen Research Institute

Procedures to be carried on: If you (your child) are willing to participate in this study, we will use your isolate for further analysis and you will be asked to answer questionnaires.

Risk and discomfort: there will be short interview which may take few minutes

Expected benefits: The result of this work can contribute to develop appropriate infection prevention and setting effective control measures to prevent the spread of this threat in the hospital and the community, help to reduces treatment failures, longer hospital stay and will also help to avoid unnecessary diagnosis costs. And if you are found positive the result will be communicated with your physician.

Confidentiality: All your personal information collected for the purpose of the present study will be kept confidential.

Payment for participation: There is no **Payment** for participation in this study.

Rights to decline participation or withdraw from the study:

Participation in the study is voluntary. You/your child are free to refuse to participate in the study or you can withdraw your consent at any time, without giving reasons and this will not involve any penalty or loss of benefits. I would also like to inform you that this study will be approved by the Departments Ethical and Review Committee and permission to conduct the research will be obtained from TASH.

Who can answer my questions about the study?

Thank you for taking time to read the information sheet/ listen as it is being read to you. If you have any questions you may ask the principal investigator or the ethics review committee.

Aminu Seman (PI) Phone: 0920747176; Email: aminu.seman@aau.edu.et or aminumifta54@gmail.com

Addis Ababa University School of Medicine;

Or you can contact ALERT /AHRI Ethical Review Committee 0113481289

B. Information Sheet in Amharic

መግቢያ

ስሜ አሚኑ ሰማን ይባላል፡፡በአዲስ አበባ ዩኒቨርስቲ የሁለተኛ ዲግሪ ተማሪ ነኝ፡፡በአሁን ሰአት የመመረቂያ ጽሁፌን ኢኤስቢል የተባለዉ መድሃኒት መላመጃ ኢንዛይም ስርጭት ማወቅ ነዉ ።

ርዕሱ: ጥቁር አንበሳ ሆስፒታል ዉስጥ ያሉ መዳሃኒትን የተላመዱ በሽታ አምጪ ተህዋስያንን ሰርጭትና መዳሃኒትን የመላመድ ባህሪያቸውንና ማጥናት ሲሆን ::

የጥናቱ አላማ፡ በዚህ ጊዜ ያሉትን መድሃኒን ከጥቅም ዉጪ የሚያደርግ ባህሪ ያላቸዉ ባክቴሪያዎችን ስርጭት ማወቅና መድሃኒቱን ከጥቅም ዉጪ የሚያደርጉበትን ኬሚካል አይነት ማወቅ ነዉ::

ጥናቱን የሚያካሂደው አካል: አዲስ አበባ ዩንቨርሲቲና አርማወር ሃንሰን የምርምር ተቋም

የ ናሙና አወሳሰድ፡ በተመራማሪዉ የተዘጋጁ አጫጭር ጥያቄዎች መመለስና ከርሶ የተገኘዉን ባክቴሪያ ለተጨማሪ ምርመራ እንድንጠቀመዉ ይፈቅዳሉ።

ሊደርሥ የ ሚችል አደጋ፡ የተዘጋጁትን ጥያቄዎች ሲመልሱ ትንሽ ሰአት እንወስትቦታለን።

ሊገኝ የሚችለው ጥቅም፡ ይህ ጥናት ወደ ፊት መሰል የሆነ ባህሪ ያላቸው ባክተሪያችን ስርጭት ለመግታትና በበሽታው ላለመያዝ የሚያስችሉ አቅጣጫዎችን ለመንደፍ ይረዳል፥ ከዚህ ጥናት ሊገኝ የሚችለው ሌላው ጥቅም ለወደፊት በበሽታው የሚያዙ ሰዎች ታክሞ አለመዳንን ለመቀነስ፥ ለረዥም ጊዜ ሆስፒታል መተኛትን ለመቀነስ ይረዳል:: የርሶ ናሙና ዉሰጥ መድሃኒት የመላመድ ባህርይ ያለው ባክቴሪያ ከተገኘ ለህኪሞ ዉጤቱ ይነገረዋል::

ሚስጥራዊነ ት፡ -ማንኛውም የጥናቱ ተሳታፊ መረጃ በሚስጥራዊነት ይያዛል፡

የተሳትፎ ክፍያ: በዚክ ጥናት በመሳተፎ ምንም አይነት ክፍያ አያገኙም

በጥናቱ ለመሳተፍ ፈቃደኛ አለመሆን ወይም ራስን የማግለል መብት በዚህ ጥናት ለመሳተፍ ሙሉ የሆነ የርሶ/የልጆ ፈቃደኝነት አስፈላጊ ሲሆን ርሶ/ልጆ በጥናቱ ያለመሳተፍ መብቶ ሙሉ በሙሉ የተከበረ ነው:: በማንኛውም ሰአት ከጥናቱ ምንም ምክንያት ማቅረብ ሳያስፈልገዎ ከጥናቱ ራስዎን ማግለል ይችላሉ::በዚህም ውሳኔ- ምንም ዓይነት ቅጣት አይጣልብዎትም ወይም ያገኙት የነበረው ህክምና አይጓደልም::

ከጥናቱ ጋር በተያያዘ ማንኛውም ጥያቄ ቢኖርዎት በሚከተሉት አድራሻዎች ጥያቄዎን ማቅረብ ይችላል::

አሚኑ ሰማን፤ የ ዋናው ተመራማሪ አድራሻ፤ ስልክ:0920747176 ኢሜይል aminu.seman@aau.edu.et
አዲስ አበባ ዩኒቨርሲቲ የህክምና ት/ቤት
አህሪ/አለርት ኢቲክስ ሪቪው ኮሚቴ ፤በስልክ ቁጥር 0113 481285 ላይ ማግኘት ይችላሉ።

Annex IIA: Informed consent form

A. For adult in English Code No:_____

Study Title: Phenotypic detection and Molecular characterization of extended-spectrum β-lactamases genes (ESBL) from clinical isolates of *Enterobacteriaceae* in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia. The Consent to participate in "Phenotypic detection and Molecular characterization of extended-spectrum β-lactamases genes (ESBL) from clinical isolates of *Enterobacteriaceae*." has been read to me/ I have read. The purpose and procedures, risks and benefits of this study have been explained to me in detail. I have been allowed to ask questions, and my questions have been answered to my satisfaction by the research staff. I have been told that I will be given a signed and dated copy of this consent form. I have been reassured that all information obtained as result of this study will be confidential and used for further study with kept confidentiality.

I understand that I have the right to withdraw from the study at any time, without any way affecting my own further medical care. If I will not participate in the study I will not involve any penalty or loss of benefits to which I am entitled.

Signature of participant: ----------Signature ----------Date ---------

Name of witness (for illiterate participant): _____Signature: _____

Name of study staff: _____Signature: _____Date: ____ / ____ / _____

Thank you for your participation!

B. Consent Form in Amharic For adult

የስምምነት ቅፅ (ከ 18 አመት በላይለ ለሆኑ)

መለያ ቁጥር----------

የጥናቱ ርእስ፡- መዳሃኒትን የተላመዱ በሽታ አምጪ ተህዋስያንን ሰርጭትና መዳሃኒትን የመላመድ ባህሪያቸውንና ማጥናት

<u>የፈቃድ መግለጫ</u>

- የዚህ ጥናት መሳተፍያ ቅጽ ያነበብኩ/የተነበበልኝ ሲሆን፣የጥናቱ አላማ፣ ናሙና አወሳሰድና ሊደርስብኝ የሚችል ችግር መኖር አለመኖሩን ተገልጾልኝ የተረዳሁ ሲሆን፡ ስለጥናቱ ጥያቄ ቢኖረኝ በማንኛዉም ሰአት እንድጠይቅ ተፈቅዶልኛል።ጥያቄዎቸም በጥናቱ ሰራተኛ ተመልሰውልኛል፡፡ ጥያቄ ካለኝ እና መወያየት ከፈለኩኝ ማንን ማግኘት እንዳለብኝ ተነግሮኛል፡፡ ስለ እኔ የተሰበሰበው መረጃ ሚስጥራዊ እንደሚሆን ተረድቻለሁ፡፡ ናሙናዉ ሚስጥራዊነቱ በተጠበቀ መልኩ ተጨማሪ የሆኑ ጥናቶች እንዲሰሩበት ተስማምቻለሁ።
- በዚህ ጥናት ውስጥ በማንኛውም ጊዜ ያለመቀጫ ወይም ጥቅም ማጣትና የእኔ የወደፊት ቀጣይ የህክምና እንክብካቤ ላይ ተጽእኖ ሳይኖር ማቆም መብት እንዳለኝ ተረድቻለሁ፡፡

የተሳታፊው ስም----------ፊርማ----------ቀን----------

የምስክር ስም (መጻፍና ማንበብ ለማይችሉ)----------ፊርማ----------ቀን----------

የመረጃ ሰብሳቢው ስም----------ፊርማ----------ቀን----------

በጥናትና ምርምሩ በመሳተፍዎ በጣም እናመሰግንዎታለን!!

C. Consent form for age less than 18 years in English

I _____being a person aged ≥ 18yrs and being the parent/Lawful guardian of_____ hereby consent to_____ in the intended research as explained and understood by me. I have understood the implications of risks and immediate benefits of the investigation (research) to _____. I have agreed _____to give the permission of processing the isolate obtained and give the answers to the questionnaires. I understand that I have the right to withdraw _____ from the research at any time, for any reason without penalty or harm. In case of withdrawal, I understand that the researchers will continue to take care of _____ like any other patient. All the above conditions have been explained to me in the language, which I can understand.

Guardian's full name _____

Guardian's signature: _____

Date: _____

Child's full name:_____

Signature of person obtaining consent_____

Witness: _____ Date: _____

D. **Consent form for age less than 18 years in Amharic**

እኔ ---------- እድሜዬ ከ18 እና ከ18 በላይ የሆነ የወጣት ---------- ---------- ወላጅህ/ጋዊ አሳዳጊ የሆንኩ ወጣት ---------- በሚቀጥለ እና በዝርዝር በተገለፀልኝ ጥናት ተሳአፊ ይሆን ዘንድ ስምምነቴን ለ---------- እሰጣለሁ። በጥናቱ ውስጥ የተካተቱ ዝርዝር አሰራሮች፣ አደጋዎች፣ ምርመራዎችና እቅዎችን ሁሉ በሚገባ የተረዳሁ ስለሆነ፣ በጥናቱ ተሳአኝ እንዲሆን ፈቅጃለሁ። በተጨማሪ፣ ወጣት----- ---------- በማንኛውም ደረጃ ከዚሁ ከተጠቀሰው ጥናት እንዲወጣ/እንድትወጣ የማድረግ ሙሉ መብት እንደሚኖረኝ ተገንዝቤአለሁ። ይህንን ለማድረግ ስወስን በወጣቱ(ቷ) ላይምንም ዓይነት ቅጣት/ጉዳት እንደማይደርስ ተገቢ የሆኑ ህክምናዎች ሁሉ ለ□□ቱ(ቷ) እንደሚደረጉ በማመን ነው። እነዚህ የስምምነት መሰረቶች ሁሉ በሚገባ በምረዳው ቋንቋ የተገለጸልኝ መሆኑን በፊርማዬ አረጋግጣለሁ።

የወላጅ/ ህጋዊ አሳዳጊ ሙሉ ስም ----------ቀን ----------ኝርማ ----------

□□□ቱ(ቷ) ሙሉ ስም ----------

□ተመራማሪ□ ሙሉ ስም ----------

□ምስ□ር ሙሉ ስም ---------- ኝርማ----------ቀን ----------

E. Assent for 12 -17 years old study volunteers in English

I _____being a person aged _____years hereby consent to_____ in the intended research as explained and understood by me. I have understood the implications of risks and immediate benefits of the investigation (research) and agreed to give the permission of processing the isolate obtained and coprate for the fulfilment of the questionnaires'. I understand that I have the right to withdraw from the research at any time, for any reason without penalty or harm. In case of withdrawal, I understand that the researchers will continue to take care of me like any other patient. All the above conditions have been explained to me in the language, which I can understand.

Name of the patient: _____

Patient's signature: _____

Data collector signature:_____Date_____

Witness: _____ Date: _____

F. Assent for 12 -17 years old study volunteers in Amharic

እኔ ---------- እትሜ□ ----------የሆነ የወጣት በሚገባ እና በዝርዝር በተገለፀልኝ ጥናት ተሳላኝ እሆን ዘንድ ስምምነቴን ለ----------እሰጣለሁ፡፡ በጥናቱ ውስጥ የተካተቱ ዝርዝር አሰራሮች፣ አደጋዎች፣ ምርመራዎችና እቁዎችን ሁሉ በሚገባ የተረዳሁ ስለሆነ፣ በጥናቱ ተሳላኝ እንድሆን ፈቅጃለሁ፡፡ በተጨማሪ፣ በማንኛ ውም ደረጃ ከዚሁ ከተጠቀሰው ጥናት እንድወጣ ሙሉ መብት እንደሚኖረኝ ተገንዝቤአለሁ፡፡ ይህንን ለማድረግ ስወስን ምንም ዓይነት ቅጣት/ጉዳት እንደማይደርስ ተገቢ የሆኑ ህክምናዎች ሁሉ እንደሚደረጉልኝ በማመን ነው፡፡ እነዚህ የስምምነት መሰረቶች ሁሉ በሚገባ በምረዳው ቋንቋ የተገለጸልኝ መሆኑን በፊርማዬ አረጋግጣለሁ፡፡

□□□ቱ(ቷ) ሙሉ ስም ----------

□ተመራማሪ□ ሙሉ ስም----------

□ምስ□ር ሙሉ ስም ---------- ኝርማ----------ቀን ----------

Annex IIIA: Questionnaire

A. English version of questionnaire

A study conducted in collaboration with Addis Ababa University and Armauer Hansen Research Institute

Study Number: _____ Study period: July to September 2018

QUESTIONNAIRES: Administered for investigation of **Phenotypic detection and Molecular characterization of extended-spectrum β-lactamases genes (ESBL) from clinical isolates of**

***Enterobacteriaceae* in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia**. We would be most grateful if you could answer the following questions.

Questionnaire code_____ Date _____Specimen type_____

S. No	Question	Answers
1	Gender	A. Male B. Female
2	How old are you?	_____ years
3	What is your Educational status?	A. Can't read and write B. Read and write C. Primary education D. Secondary education E. College or University
4	Do you have been taken antibiotics in the past 3 month?	A. Yes B. No
5	If the answer is 'yes' for question number 5 what are these?	Cephalosporin Aminoglycoside Quinolones Folate pathway inhibitor Carbapenems Beta-lactam inhibitor
6	Have you been admitted to hospital in the last 3 month?	A. Yes B. No
7	If 'yes' for question number 6 for how long?	<7 days 7-14 days 15-29 days >30 days
8	Have you been admitted to intensive care unit ward in the last 3 month?	A. Yes B. No
9	If 'yes' for question number 8 for how long?	<3 days 4-7 days 8-12 days >15 days
10	Do you have underlying medical conditions?	A. Yes B. No
11	If 'yes' for question number 11 which one from the listed	DM HTN Cancer Renal disease Cardiac disease Chronic viral diseases (heptits,HIV….) Genetic disorder
12	Do you have medical device?	A. Yes B. No
13	Do you have a history of surgery in the past 3 month?	A. Yes B. No
15	Are you admitted yet?	A. Yes B. No
16	If 'yes' for question number 15 when did you	

	get admitted here and for how long you stayed here?		
17	If ‘yes’ for question number 15 in which ward you are admitted?		

B. Amharic version of questionnaire

ከአዲስ አበባ ዩኒቨርሲቲ እና ከአርማወር ሐንሰን የምርምር ተቋም ጋር በመተባበር የሚደረግ ጥናት ነዉ

የጥናቱ መለያ ቁጥር _____ ጥናቱ የሚካሄድበት ጊዜ፡ ከሃምሌ 2010-መስከረም 2011 ዓ.ም

መጠይቁ በአዲስ አበባ ከተማ በጥቁር አንበሳ ስፔሻላይዝድ ሆስፒታል የመጡ ታካሚዉች ላይ የተገኙ ኢኒቴሮባክቴሪያሴ ባክቴሪያዎችን መዳኒትን የመቋቋም ዉጫዊ እና ዉስጣዊ ባህሪያቸዉን ለማወቅ የሚደረግ ጥናት ነዉ፡፡ የሚከተለዉን መጠይቅ ቢሞሉልን በጣም ደስ ይለናል፡፡

የመረጃ ማሰባሰቢያ ቅጽ ኮድ_____ ቀን_____የናሙና አይነት_____

ተ.ቁ	ጥያቄ	መልስ	ምርመራ
1	ጾታ	ሀ. ወንድ ለ. ሴት	
2	እድሜህ/ሽ ስንት ነዉ?	_____አመት	
3	የትምህርት ደረጃዎ ምን ያክል ነው?	ሀ. ማንበብና መፃፍ አልችልም ለ. ማንበብና መፃፍ አችላለሁ ሐ. አንደኛ ደረጃ መ. ሁለተኛ ደረጃ ሠ. ኮሌጅ ወይም ዩኒቨርሲቲ	
4	ባለፉት ሶስት ወራት ጸረ-ባክቴሪያ መድሃኒት ወስደው ያውቃሉ?	ሀ. አዎ ለ. የለም	
5	ለጥያቄ ቁጥር 5 መልስዎ ‘አዎ’ ከሆነ ምን ምን ናቸው ?	ሀ ሴፉሎስፖሪን ለ አሚኖግላይኮሳይድ ሐ ኪኖሎንስ መ ፎሌትፓዝዌ ኢንሂቢተር ሠ ካርባፔኔም ሸ ቤታላክታም ኢንሂቢተር	
6	ከሶስት ወር ወዲህ ሆስፒታል ተኝተው ያውቃሉ ?	ሀ. አዎ ለ. የለም	
7	ለጥያቄ ቁጥር 6 መልስዎ ‘አዎ’ ከሆነ መቼ ?	ሀ <7 ቀናት ለ 7-14 ቀናት ሐ 15-29 ቀናት መ >30 ቀናት	

8	የጽኑ ህመማን ማቆያ ክፍል ተኝተው ያውቃሉ?	ሀ. አዎ ለ. የለም	
9	ለጥያቄ ቁጥር 8 መልስዎ 'አዎ' ከሆነ መቼ?	ሀ <3 days ለ 4-7 days ሐ 8-12 days መ >15 days	
10	ሌላ አይነት ህመም አለብዎት?	ሀ. አዎ ለ. የለም	
11	ለጥያቄ ቁጥር 10 መልስዎ አዎ ከሆነ ምን አይነት ?	ሀ ስኳር ለ የደም ግፊት ሐ ካንሰር መ የኩላሊት በሽታ ሠ የልብ ህመም ሸ ስርየሰደደ የቫይረስ ብሽታ (heptits,HIV….) ቀ የዘረመል ችግር	
12	ሰውነትዎ ላይ የገባ የህክምና መሳሪያ አለ?	ሀ. አዎ ለ. የለም	
13	በዚህ 3 ወር የቀዶ ጥገና ህክምና አድርገው ያውቃሉ ?	ሀ. አዎ ለ. የለም	
14	ለጥያቄ ቁጥር 13 መልስዎ 'አዎ' ከሆነ መቼ?		
15	ተኝቶ ታካሚ ነዎት?	ሀ. አዎ ለ. የለም	
16	ለጥያቄ ቁጥር 15 መልስዎ 'አዎ' ከሆነ ምን ያክል ቆይተዋል?		
17	ለጥያቄ ቁጥር 16 መልስዎ 'አዎ' ከሆነ ምን ክፍል ዉስጥ?		

Annex IVA: Laboratory activities

1. Gram's stain preparation

Gram Staining is the common, important, and most used differential staining techniques. This test differentiates the bacteria into Gram Positive, appears blue or purple and Gram Negative, appears red or pink color, which helps in the classification and differentiations of microorganisms.

Reagents Used and Materials Required

- Crystal Violet, the primary stain
- Iodine, the mordant
- A decolorizer made of acetone and alcohol (95%)
- Safranin, the counterstain
- Clean glass slides
- Inoculating loop
- Bunsen burner
- Bibulous paper
- Microscope
- Immersion oil
- Distilled water
- 18 to 24 hour cultures of organism

Procedure of Gram Staining

1. Labeling the slides clearly with patient code number
2. With a sterile cooled loop, place a loopful of the culture on the slide. Spread by means of circular motion of the inoculating loop to about one centimeter in diameter.
3. Air dry and heat fix
4. Place slide with heat fixed smear on staining tray.
5. Gently flood smear with crystal violet and let stand for 1 minute.
6. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
7. Gently flood the smear with Gram's iodine and let stand for 1 minute.
8. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
9. Decolorize using 95% acetone alcohol. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear.
10. Immediately rinse with water.

11. Gently flood with safranin to counter-stain and let stand for 45 seconds.
12. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
13. Blot dry the slide with bibulous paper.
14. View the smear using a light-microscope under oil-immersion.

- Gram Positive: **Blue/Purple Color**
- Gram Negative: **Red/pink Color**

2. Biochemical test

For identification of *Enterobacteriaceae* we have used the following biochemical tests medium.

- Tryptophan: helps to see production of tryptophanase enzyme
- Urea: shows the ability of the isolates to hydrolyze urea by producing the enzyme urease
- Triple Sugar Iron: which has multi-purposes, helps to see fermentation of Carbohydrates, production of gas and H_2S
- Citrate: which is essential to see bacterial ability to utilize it as sole carbon source
- Lysine decarboxylase: lysine get decarboxylated when Enterobacteriaceae isolate produce Lysine decarboxylase enzyme
- Motility: helps to see the ability of an organism to move by itself by means of propeller-like flagella
- Malonate tests medium: was used to test the ability of the organism to utilize malonate as sole source of carbon.

Procedure

1. Prepare a 0.5 McFarland suspension of the test organism with nutrient broth.
2. A loop full of the bacterial suspension is inoculated in tryptophan, urea, triple sugar iron, citrate, lysine decarboxylase, motility and mannitol tests medium.
3. Incubate at $37^{O}c \pm 2^{0}C$ for 18-24 hours
4. Look for production of indole by adding Kovacs reagent in tryptophan broth
5. Look for color change in the other test medium (turbidity for motility) of the medium
6. Identify the test organism by following standard biochemical test result chart for Enterobacteriaceae.

3. Antimicrobial Susceptibility Testing

Method: Modified Kirby-Bauer susceptibility testing technique

Requirements: Mueller Hinton agar, Antimicrobial discs, McFarland machine, and Control strains to test performance standard of Antimicrobial discs

4. Biochemical test

For identification of *Enterobacteriaceae* we have used the following biochemical tests medium.

- Tryptophan: helps to see production of tryptophanase enzyme
- Urea: shows the ability of the isolates to hydrolyze urea by producing the enzyme urease
- Triple Sugar Iron: which has multi-purposes, helps to see fermentation of Carbohydrates, production of gas and H_2S
- Citrate: which is essential to see bacterial ability to utilize it as sole carbon source
- Lysine decarboxylase: lysine get decarboxylated when Enterobacteriaceae isolate produce Lysine decarboxylase enzyme
- Motility: helps to see the ability of an organism to move by itself by means of propeller-like flagella
- Malonate tests medium: was used to test the ability of the organism to utilize malonate as sole source of carbon.

Procedure

7. Prepare a 0.5 McFarland suspension of the test organism with nutrient broth.
8. A loop full of the bacterial suspension is inoculated in tryptophan, urea, triple sugar iron, citrate, lysine decarboxylase, motility and mannitol tests medium.
9. Incubate at $37^{O}c \pm 2^{0}C$ for 18-24 hours
10. Look for production of indole by adding Kovacs reagent in tryptophan broth
11. Look for color change in the other test medium (turbidity for motility) of the medium
12. Identify the test organism by following standard biochemical test result chart for Enterobacteriaceae.

5. Antimicrobial Susceptibility Testing

Method: Modified Kirby-Bauer susceptibility testing technique

Requirements: Mueller Hinton agar, Antimicrobial discs, McFarland machine, and Control strains to test performance standard of Antimicrobial discs.

Procedure:

1. Using a sterile wire loop, prepare 0.5 McFarland standard suspension in sterile physiological saline or nutrient broth.

2. Using a sterile swab, inoculate a plate of Mueller Hinton agar. Streak the swab evenly over the surface of the medium in three directions, rotating the plate approximately 60^{o} to ensure even distribution.
3. With the petri dish lid in place, allow 3-5 minutes (*no longer than 15 minutes*) for the surface of the agar to dry.
4. Using sterile forceps, needle mounted in a holder, or a multidisc dispenser, place the appropriate antimicrobial discs, evenly distributed on the inoculated plate.
   Note: The discs should be about 15 mm from the edge of the plate and no closer than about 25 mm from disc to disc. No more than 6 discs should be applied (90 mm dish). Each disc should be lightly pressed down to ensure its contact with the agar. It should not be moved once in place.
5. Within 30 minutes of applying the discs, invert the plate and incubate aerobically at 35^{o}C for 16-18 hours.
6. After overnight incubation, examine the control and test plates to ensure the growth is confluent or near confluent. Using caliper on the underside of the plate measure the diameter of each zone of inhibition in mm.

Interpretation of zone sizes:

Based on CLSI criteria, interpret the zones sizes of each antimicrobial, reporting the organism as 'Resistant', 'Intermediate/Moderately susceptible', 'Susceptible'.

6. ESBL confirmation

Method: combined disk diffusion technique

Requirements: suspected isolate, Muller hinton agar, discs containing cephalosporin alone (cefotaxime, ceftazidime, cefepime) and in combination with clavulanic acid and control strains to test effectiveness of the disks.

TEST PROCEDURE

1. Using a fresh, pure culture prepare a suspension of the test organism equal to 0.5 McFarland Standard.

2. Using a sterile cotton swab, spread the adjusted suspension over the entire area of a Mueller Hinton agar plate.
3. Apply the discs onto the inoculated plate, ensuring sufficient space between individual discs to allow for proper measurement of inhibition zones.
4. Incubate at 35±2°C for 18-24 hours.
5. At the end of the incubation period the inhibition zone around the cephalosporin disc combined with clavulanic acid is compared with the zone around the disc with the cephalosporin alone. The test is positive if the inhibition zone diameter is ≥ 5 mm larger with clavulanic acid than without.

Annex VI: **Declaration**

Declaration

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

Principal investigator: Aminu Seman (BSc, MSc candidate)

signature_____, Date of submission_____

This thesis has been submitted with our approval as advisors.

Name of supervisor Signature

Tamrat Abebe (PhD, Assistant Professor) _____

Daniel Asrat (MD, PhD, Professor) _____

Magnitude of Extended Spectrum Beta-lactamase producing Multidrug resistant *Enterobacteriaceae* isolates from patients attending Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia.