Genomic Epidemiology and Molecular Features of Extended Spectrum β-lactamase-producing *Enterobacteriaceae* at Jimma Medical Center, Ethiopia.

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A PhD thesis submitted to the Department of Microbiology, Immunology and Parasitology (DMIP), College of Health Sciences, Addis Ababa University in partial fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) in Medical Microbiology

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Genomic Epidemiology and Molecular Features of Extended Spectrum β-lactamase-producing *Enterobacteriaceae* at Jimma Medical Center, Ethiopia

By

Tsegaye Sewunet Wondimu

A Thesis Presented to the School of Graduate Studies of Addis Ababa University for the Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medical Microbiology

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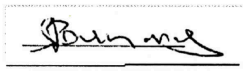
Prof. Spyros A. Pournaras External Examiner		July 27, 2020
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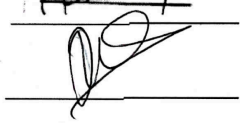
Prof. Daniel Asrat Advisor		July 27, 2020
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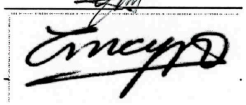
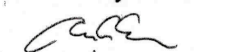
Dr. Abraham Aseffa Advisor		July 27, 2020
Prof. Christian G. Giske Advisor		July 27, 2020
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ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
AmpC	Ampicillinase
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
BEL	Belgium Extended spectrum β-lactamase
BES	Brazilian Extended Spectrum β-lactamases
βL/βLI	β-lactam and β-lactam inhibitor
cgMLST	Core-genome multi-locus sequence typing
CLSI	Clinical Laboratory Standards Institute
COPD	Chronic Obstructive Pulmonary Disease
CRE	Carbapenemase Resistant *Enterobacteriaceae*
CTX-M	Cefotaximase Munich
ESBL	Extended Spectrum β-Lactamase
EUCAST	European Committee of Antimicrobial Susceptibility Testing
GES	Guyana Extended spectrum β- lactamases
ICU	Intensive Care Unit
ISCR	Insertion sequence common region
IV	Intravenous
KL	Capsular Locus
KPC	*Klebseilla Pneumoniae* Carbapenemase
LPS	Lipopolysaccharides
MDR	Multidrug resistant
MGE	Mobile Genetic Elements
MLST/ST	Multi-Locus Sequence Typing/Sequence type
MRSA	Methicillin Resistant *Staphylococcus aureus*
NGS	Next Generation sequencing
OXA	Oxacillinase
PCR	Polymerase Chain Reaction

PDR	Pandrug-resistant
PER	Pseudomonas Extended Resistance
PFGE	Pulsed-Field Gel Electrophoresis
RFLP	Restriction Fragment Length Polymorphism
rMLST	Ribosomal multi locus sequence typing
SFO	Serratia Fonticola (ESBL from *Serratia fonticola*)
SHV	Sulfhydryl variable variant ESBL
SNP	Single Nucleotide polymorphism
TEM	Temoniera variant ESBL
UTI	Urinary tract infection
VEB	Vietnam Extended spectrum β-lactamases
VIM	Verona integron-encoded metallo-β-lactamase
wgMLST	Whole-genome multi-locus sequence typing
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR	Extensive drug resistant

ABSTRACT

Introduction: Infections caused by *Enterobacterales* are common causes of morbidity and mortality in hospitals and community. Several species of the *Enterobacterales* emerged with resistance property to extended/broad-spectrum β-lactam antimicrobials. Such strains produce β-lactamase enzymes that can inactivate extended spectrum β-lactam antimicrobials, and hence, are called Extended Spectrum β-lactamase (ESBL)-producing strains. ESBL-producing strains are increasingly being reported from different parts of the world revealing increased prevalence and shift of predominant molecular types over last two decades. However, there is a limited number of phenotypic studies in Ethiopia. The studies are not conclusive enough to unravel the magnitude, dissemination, and the actual burden of the problem.

Objective: The study aimed to determine genomic epidemiology and molecular features of ESBL-producing *Enterobacteriaceae* at Jimma Medical Center (JMC), Ethiopia.

Methods: A cross-sectional study was conducted at JMC from June-October 2016. Study participants were identified with the help of clinicians. And specimens relevant to clinical diagnosis of the patients were collected by experienced nurses. Specimens collected included: urine, stool, wound-swab/aspirates, and sputum. Then specimens processed and inoculated on MacConkey agar, Blood agar and Salmonella Shigella agar at the microbiology laboratory and incubated at 37^{o}C for 18-24h. Species identification was performed primarily by conventional biochemical tests.

Phenotypic screening and confirmation of ESBL-production was determined by Double Disc Synergy Test (DDST). Further validation/confirmation of species identification and antibiotic susceptibility test was performed by MALDI-TOF, and disk diffusion for ESBL-phenotype at Karolinska University Hospital, Department of Clinical microbiology, Sweden. Strains of *E. coli* and *K. pneumoniae* with ESBL-phenotype were selected for whole genome sequencing (WGS) and sequenced on the Illumina (HiSeq 2500) sequencing platform at *SciLifeLab*, Stockholm, Sweden.

Socio-demographic, clinical data and associated variables were collected using pre-designed structured questionnaire. Ethical clearance was obtained from relevant bodies. Study subjects

were given information sheet about the study. Moreover, only study participants who provided written consent were recruited for the study.

Descriptive data analysis was performed by using SPSS ver.20 statistical software. Socio-demographic, clinical data, and species related proportions were summarized by descriptive parameters. Where applicable, statistical association and significance was defined as p-value ≤ 0.05 as cut-off value for statistical association. Genome assembly and analysis was performed by using different bioinformatics tools, SPAdes *version 3.9* was used for genome assembly. Enterobase, Center for Genomic Epidemiology (CGE), BIGsdb (**B**acterial **I**solate **G**enome **s**equence **d**ata**b**ase), and Gubbins (**G**enealogies **U**nbiased **B**y recom**B**ination **I**n **N**ucleotide **S**equences), FastTree v.2, iTOL were tools used for genomic data analysis.

Results: A total of 1,087 study participants either admitted to wards or seeking care as out-patient at medical, surgical, pediatric, and intensive care units at JMC were recruited. From these study participants, 58.9%(n= 640) were male and 41.1%(n=447) were female. A total of 1,087 specimens were collected, and 642 bacterial strains of different spp. were isolated. Most, 63.8% (n=255) were either *E. coli*(n=144) or *Klebsiella spp*.(n=111) and among non-fermenters, *Pseudomonas aeruginosa* and *Acinetobacter spp*. were predominant and accounted for 13% (n=87) of overall isolates. Within the four main units of the hospital, and among all specimens collected, *E. coli* and *Klebsiella spp.* were the most common strains isolated.

The prevalence of ESBL-producing strains was (54.9%, 144/262) among *E. coli* and (76%, 111/146) among *K. pneumoniae*. A small proportion of *E. coli* strains (5.4%, 14/262) were non-susceptible to carbapenems. But only two *E. coli* strain encoded carbapenemaseng gene ($bla_{\text{NDM-1}}$ and $bla_{\text{OXA-66}}$). Similarly, a total of (22.6%, 33/146) of *K. pneumoniae* strains were non-susceptible to carbapenems, however, only one strain encoded $bla_{\text{NDM-1}}$. Moreover, *E. coli* 96% (136/141) and *K. pneumoniae* 92%(102/111) were resistant to at least three classes of antimicrobials.

Several genetic determinants of antimicrobial resistance were identified, where $bla_{\text{CTX-M-15}}$ was the most prevalent ESBL, *E. coli* (88.4%, 123/139) and *K. pneumoniae* (84.4%, 92/109). The other genetic determinants included: $bla_{\text{TEM-1B}}$ (53.9%, 75/139) , $bla_{\text{OXA-1}}$ (63.3%, 88/139) , *dfrA17* (58.2%, 81/139), *mph* (67.2%, 94/139), *aac(6')-Ib-cr* (62.6%, 87/139), and *sul* (68.3%, 95/139) among *E. coli* strains, and $bla_{\text{TEM-1B}}$ (69.7%, 76/109), $bla_{\text{OXA-1}}$ (34.8%, 38/109), *sul*

(61.5%, 67/109), *aac(6')-Ib-cr* (65.1%, 71/109), and *dfrA27* (32.1%,35/109) among *K. pneumoniae* strains.

Multi-locus sequesnce typing (MLST) revealed several sequence types (STs) carrying the ESBL genes. The most common sequence types (ST) encoding the $bla_{\text{CTX-M-15}}$ were ST410 (20/21), ST648 (15/15), ST131 (8/8), ST38 (6/6), ST44 (4/4) among *E.coli*. Among *K. pneumoniae* ST218 (7/7), ST15 (6/6), ST147 (6/6), ST17(6/6) and ST39 (5/5) had highest carriage of ESBL-genes.

In both *K. pneumoniae* and *E. coli* strains, virulence determinant genes were invesitigated. EPEC, ExPEC/UPEC, EAEC were the prevalent pathotypes identified among *E. coli* strains. The O15/99:H30-ST38 (100%, n=6), O25:H4-ST131 (100%, n=8), O176:H34-ST130 (100%, n=5) encoded multiple toxins/virulence genes. On the other hand, *K. pneumoniae* strains were characterized by capsular and O-LPS antigens/types. Both classical and hypervirulent strains were identified as multi-drug resistant (MDR) strains carrying $bla_{\text{CTX-M-15}}$ ESBL-genes.

Plasmid replicon typing was performed, *IncFII*(*pRSB107*) (24.8%, n=32), *IncQ1*(20%, n=26) and *Col*(*BS512*) (14%, n=18) are the most commonly identified replicons among *E. coli* strains, and *IncFIB*(*K/Mar/PQil*) (32.9%, n=32), *IncR* (24.7%, n=24), and *IncFII* (14.4%, n=14) were prevalent replicons among *K. pneumoniae* strains.

Single nucleotide polymorphysim (SNP)-based phylogenetic analysis of population structure showed multiple clusters of related strains. Though $bla_{\text{CTX-M-15}}$ is the predominant ESBL-gene, other genetic determinants of resistance to β-lactams and other classes of antimicrobials were identified with a higher proportion in the same clonal complexes. Furthermore, some of these strains (both in *E. coli* and *K. pneumoniae)* were high-risk epidemic clones. The dissemination of $bla_{\text{CTX-M-15}}$ mediated antimicrobial resistance was thus polyclonal. Some of the detected conal clusters suggest that an outbreak might have likely occured in the hospital.

Furthermore, some possible risk factors for the increased prevalence of ESBL-strains were studied. Among these factors age less than five years, admission, pediatric unit and presence of underlying chronic illness showed statistically significant association with the higher prevalence of ESBL-strains. Gender and current use of antibiotics (reported use of antibiotics) showed no statistically significant association

Conclusion: very high prevalence of ESBL among *E. coli* and *K. pneumoniae* was observed. The *bla*$_{\text{CTX-M-15}}$ was the predominat ESBL-genetic variant. Most of the strains are MDR, also resistant to several other non-β-lactam antimicrobials. The detection of *bla*$_{\text{NDM-1}}$ is a clear threat of evolving carbapenemases mediated carbapenem resistance. The spread of ESBLs is through multiple clusters of epidemic clones, and some of them are hypervirulent strains. The findings of this study are exteremly important primarly for the current hospital to look into it's practice, to look into immediate preventive strategies including implemention of strict antimicrobial stewardship programmes and infection control strategies. The findings also shed light on genomic diversity and transmission dynamics of ESBL strains from Ethioipia and hence serve as a baseline data for researchers, planners and policy makers.

Key words: ESBL, antimicrobial resistance, *E. coli*, *K. pneumoniae*. Jimma, Ethiopia.

CHAPTER 1: INTRODUCTION

1.1 General Introduction

Bacteria, prokaryotic organisms, express biochemical substances that help them to adapt to their environment (Rennie, 2012). These biochemical substances were exploited for human benefits in many ways. Some of the benefits in science include enzymes used in biotechnology and use as antimicrobial agents. However, by similar mechanisms, many medically important pathogens have been continually evolving to a new or modified property by which they develop resistance to commonly prescribed antimicrobial agents (M'Zali *et al*., 2000).

Gram-negative bacilli were long understood as cause of several infections in both humans and animals. *Enterobacterales* and non-fermentative Gram-negative bacilli like, *P. aeruginosa* and *A. baumannii* can cause a variety of diseases ranging from community-acquired to hospital-acquired infections (Giamarellou, 2005; Herindrainy *et al*., 2011; Potron *et al*., 2015; Rawat and Nair, 2010).

The severity and magnitude of infections caused by these organisms vary based on overall conditions of the patient. However, study show that lower respiratory tract infections and bloodstream infections are the most lethal whereas urinary tract infection is the most common (Giske *et al*., 2008). Gram-negative bacilli are highly efficient at up-regulating or acquiring genes that encode antimicrobial resistance usually when exposed to antibiotics. They have a plethora of resistance mechanisms, often using multiple mechanisms against the same antibiotic or using a single mechanism to affect multiple antibiotics. The presence of multiple virulence factors together with their versatile property of antimicrobial resistance allow these bacteria to persist in the existence of an antimicrobial agent which was lethal to them before (Heritage *et al*., 1999).

Antimicrobial agents are critical for treatment of bacterial infections and overall improvement of medical care. Discovery of penicillin was followed by improvement in medical care and progress in research related to antibiotics (Donowitz and Mandell, 1988). Excessive use of these antibiotics in turn resulted in the emergence of bacterial strains resistant to penicillin. The resistant strains developed a survival advantage under the selective pressure of antibiotics

over susceptible strains. The resistant strains propagated and spread throughout the world (Giske *et al*., 2008; Kapil, 2005). Consequently, magnitude and severity of diseases caused by resistant strains increased. The cost of treatment for an infection with resistant strains increased rapidly. The threat of antibiotic resistance has become global problem due to rapid spread of organisms from one part of the world to another (Isturiz, 2008).

Antimicrobial resistance has been recognized a priority problem of both low-income and high-income countries, and one of the priority agendas of WHO (Furyk *et al*., 2011). Human to human transmission of resistant bacterial strains is documented among hospital acquired, and community acquired infections. The incidence and prevalence of morbidity and mortality from infection with resistant bacterial strains is increasing in Europe and US (Colomb-Cotinat *et al*., 2016).

In Gram-negative bacilli, β-lactamase production remains the most important mechanism for β-lactam antimicrobial resistance (Bradford, 2001). β-lactamases are bacterial enzymes that inactivate β-lactam antibiotics by hydrolysis. One group of β-lactamases, extended-spectrum β-lactamases (ESBLs) have ability to hydrolyze and inactivate third-generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime), and monobactams (aztreonam) but not the cephamycins (e.g. cefoxitin and cefotetan) and carbapenems (e.g. imipenem, meropenem, and ertapenem) (Karen and Jacoby, 2010). Hydrolysis of the core structure (the β-lactam ring) of β-lactam antimicrobial agents inactivate the compounds. Treatment and/or medical care strategies using these agents in the presence of inactivating enzymes is ineffective.

Gram-negative bacilli mainly *Enterobacterales* and non-fermentative strains (*P. aeruginosa and A. baumannii*) are groups of bacterial agents causing several infections worldwide and also resistant to important class of antimicrobial agents used for treatment of infections (Haeili *et a*l., 2013; Menichetti and Tagliaferri, 2012; Rennie, 2012). ESBL-producing *Enterobacterales* particularly *E. coli* and *K. pneumoniae* have become a global problem. The knowledge of prevalence, severity and other epidemiologic data is highly required for improving clinical care and public health. Genomic studies uncovered molecular features of β-lactamase enzymes, dissemination and genetic relatedness of strains with ESBL phenotype that vary from local to global (Paterson, 2006b). Several studies reported detection of genetic determinants of ESBL

enzymes from a number of *Enterobacterales* isolates including, *Klebsiella pneumoniae (K. pneumoniae), Klebsiella oxytoca (K. oxytoca), Escherichia coli (E. coli), Proteus mirabilis (P. mirabilis), Enterobacter cloacae (E. cloacae), Morganella morganii (M. morganii), Serratia spp.,* and *Salmonella spp*. (Dhillon and Clark, 2012; Rupp and Fey, 2003; Paterson, 2006a; Yu *et al.*, 2006).

Primarily, infections caused by ESBL-producing bacteria may cause higher rate of patient hospitalization and longer hospital stay, also increased cost of treatment, and when an infection from ESBL-producing bacteria is super-imposed on the already existing illness; it may further worsen prognosis of the already existing illness. Moreover, increased rate of resistant strains being reported from surgical site infections signal the challenge on the effectiveness and prognosis of surgical procedures. Antimicrobial resistant strains are often reported to cause community-acquired and/or community onset and hospital-acquired infections. These ESBL-producing strains were also reported among immunocompromised study populations including malignancy and AIDS.

Though burden of antimicrobial resistance is a global problem, magnitude of the problem is much larger in low-income countries (Kapil, 2005). It imposes unbearable burden on the already compromised economy of these countries and further compromising health care delivery of these nations (Wilke, 2010). Though there were few studies conducted in low-income countries, it can be observed that there are many factors that may contribute to an increased prevalence of resistance strains. Of these factors, combination of a high disease burden, huge populations, crowding, poor sanitation, inappropriate use of the available drugs, and veterinary use of drugs are major factors that contributed to the problem (Arnold and Straus, 2005; Okeke *et al*., 2007).

Tackling the problem of antimicrobial resistance should start with generating comprehensive epidemiologic data from local to national. Cooperation with global alliances and strategies against antimicrobial resistance is desirable, and most importantly comprehending available local data for local consumption is necessary. In our setting, there is limited data regarding epidemiology of antimicrobial resistance. In Ethiopia, there are few phenotypic studies, these studies lack the depth required to understand epidemiology of ESBL strains in both clinical

and community setting. Therefore, this study was undertaken to shed light on epidemiology and molecular characteristics of ESBL-producing strains isolated at Jimma Medical Center, Ethiopia, a prominent tertiary health care facility providing service for 15 milion population in one of the densely populated regions of South-west Ethiopia.

1.2 Literature review

1.2.1 General microbiologic characteristics of *Enterobacterales*

Enterobacterales, Gram-negative rod-shaped organisms are generally 1-3μm in length and 0.5μm in diameter. Surface appendages including pili and flagella are common and may be numerous. *Enterobacterales* are non-spore forming, facultative anaerobes that ferment glucose and other sugars, reduce nitrate to nitrite, and produce catalase. Most are motile by peritrichous flagella and *Enterobacterales* do not produce oxidase.

Members of the family *Enterobacterales* are often referred to as enteric bacteria because the principal habitat of many of these organisms is the lower gastrointestinal tract of humans and animals. However, the term 'enteric bacteria' is not synonymous with *Enterobacterales.* There are several species of Gram-negative bacilli that do not typically inhabit the gastrointestinal tract, and other Gram-negative bacteria that dwell in intestine/are intestinal pathogens that do not fall within the family *Enterobacterales*, such as *Vibrio* spp., that are also referred to as enteric bacteria. The use of this term also compromises other members of the family *Enterobacterales* that are widely distributed and commonly found in the environment (Donnenberg, 2009). Although the natural habitat of many medically important members of the *Enterobacterales* is the lower gastrointestinal tract of humans and other animals; these organisms are also widespread in nature. The extended niche that *Enterobacterales* may occupy under different circumstances is an important feature that allows subsequent extra-intestinal infections. Members of the family *Enterobacteriaceae* cause a wide variety of infections in both community and clinical setting affecting both healthy hosts and those with preexisting illnesses.

The proportion of multiple antimicrobial resistant *Enterobacterales* isolates, including those producing ESBL and resistant to fluoroquinolones, has increased steadily, so that the majority

of nosocomial and community acquired isolates are now resistant to several important antimicrobial classes. Infections caused by members of this family may be sporadic or occur in outbreaks. Detection of source of outbreak as community acquired or nosocomial acquired demand tools with very high sensitivity and specificity. The use of molecular methods for diagnosis including whole genome sequencing are tools of choice for detection and tracking source of infection. For example, to rule out whether strains isolated from different patients arose from a recent common ancestor (i.e., belong to the same clone) (Donnenberg, 2009).

1.2.2 Classifications of *Enterobacterale*s

Historically, bacteria were once grouped under Kingdom Plantie, but when advances in techniques and technologies revealed more infromation about microorganisms, and these advances in knowledge were used to develop better taxonomy and nomenclature of organisms than before, bacteria were later defined in separate kingdom called Kingdom Monera. However, in bacterial taxonomy there are yet complexities in classification at each level of the taxa. Recently the most complex order '*Enterobacteriales'* was re-defined as order *Enterobacterales* based on genomic phylogeny. The ord. novus. *Enterbacterales* is classified into new seven different Families, and few Genuses. The new classification encompass the amended family *Enterobacteriaceae*, the fam. nov. *Erwinaceae*, *Pectobacteroaceae*, *Yersiniaceae*, *Hafiniaceae*, *Morganellaceae*, and *Budviciaceae*. Bacterial species that belong to genus *Buchnera*, *Plesiomonas*, and *Wigglesworthia* previously classified under the family '*Enterobacteriaceae*' were grouped in separate genus under the order *Enterobacterales* (Adeolu *et al*., 2016).

I. Family *Enterobacteriaceae*

Genus *Escherichia*: *E. coli* is the most common species of facultative anaerobe found in the human gastrointestinal tract and the most commonly encountered pathogen from the family *Enterobacteriaceae*. Large amount of information is available regarding the genetics, structure, and physiology of this organism, and further studies are required to fully characterize the biochemistry and cell biology of both pathogenic and non-pathogenic strains. *E. coli* is usually distinguished from other members of the family by its ability that most strains ferment lactose

and other sugars and to produce indole from tryptophan; most strains of these species are also motile (Friedman *et al*., 2002; Welch *et al.*, 2002).

E. coli exhibits versatility in its ability to cause disease and bears different mechanisms by which it causes specific diseases. Pathogenic strains differ from commensal organisms in that they produce virulence factors specific for each pathotype, which may be encoded by bacteriophages, plasmids, or on stretches of the chromosome known as pathogenicity islands (Napolitano *et al*., 2011). Comparisons among the fully sequenced genomes of nonpathogenic and pathogenic strains have revealed an average genome size of approximately 5000 genes, but only approximately 2200 of these are shared among all *E. coli* strains (Rasko *et al*., 2008; Wick *et al*., 2005). Most of the pathogens have larger genomes than do the non-pathogenic strains (Blattner *et al*., 1997). Furthermore, many of the genes that are not found in the non-pathogenic strain are specific to particular strains of pathotypes (Welch *et al*., 2002)

Nowadays, there are several pathotypes of *E. coli* that can cause specific types of illness in healthy hosts and those with compromised defense mechanisms. Of these pathotypes, the more characterized strains include Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), Diffuse adhesive *E. coli* (DAEC) (Rasko *et al*., 2008; Simona Santona *et al*., 2013; Toval *et al*., 2014; Zeighami *et al*., 2015).

The classical classification of *E. coli* strains into different pathotypes is based on the pathology of infected intestinal cells. However, with the advances in molecular diagnostics, in the era of whole genome sequencing, virulome/virotypes are usually defined by specific combinations of genes. In this regard, a sub-class of Enteropathogenic *E. coli* was distinctly studied or designated as Extra-intestinal pathogenic *E. coli* (ExPEC). ExPEC strains were defined on the bases of virulence genes they encode (*pap*A/*pap*C*, sfa/foc, afa/dra iutA, and kpsM II*). ExPEC is therefore, defined as positive for ≥2 of *papA* and/or *papC* (P fimbriae counted as one), *sfa/foc* (S fimbriae and F1C fimbriae), *afa/dra* (Dr-binding adhesins), *kpsM II* (group 2 capsule) and *iutA* (aerobactin system) (Boisen *et al*., 2012).

Genus *Klebsiella*: Several species of the genus *Klebsiella* are associated with illness in humans. These species include: *Klebsiella pneumoniae, Klebsiella oxytoca,* and *Klebsiella granulomatis*. But, recently strains being reported as *K. pneumoniae* were further classified in to three distinict phylogenetic groups KpI (*K. pneumoniae*), KpII (*K. quasipneumoniae*), and KpII (*K. variicola*); and hence independent, but related species of the genus, the *Klebsiella* (*K. pneumonae*, *K. variicola*, and *K. quasipneumoniae*) (Holt et al., 2015). Organisms previously known as *Klebsiella ozaenae* and *Klebsiella rhinoscleromatis* are considered non-fermenting sub-species of *K. pneumoniae* that have characteristic clinical manifestations. With those exceptions, strains within this genus ferment lactose and most produce highly mucoid colonies on plates because of the production of a dense polysaccharide capsule, and all are non-motile (Donnenberg MS, 2009). Moreover, a bacterial species previously called *Enterobacter aerogens* was redefined by the International Committee on Systematics of Prokaryotes(ISCP) as *K. aerogenes* and now officially included in the genus *Klebsiella* (Tindall *et al*., 2017), although the various properties of this species resembles *Enterobacter* more than *Klebsiella*.

K. pneumoniae as a pathogen can cause UTIs, liver abscess, and pneumonia. Most infections caused by *K. pneumoniae* are acquired in hospital settings among patients who are debilitated by various underlying conditions. Most prevalent nosocomial infections caused by *K. pneumoniae* were pneumonia and UTIs. However, nosocomial infections caused by *K. pneumoniae* also include wound infections, intravascular/other invasive-device related infections, biliary tract infections, peritonitis, and meningitis. Hypervirulent strains of *K. pneumoniae* can also cause serious infections among apparently healthy individuals with otherwise healthy and non-immune-compromised individuals. This bacterium has become one of the common causes of bacteremia resulting from UTI among other causes of Gram-negative bacteremia (Podschun and Ullmann, 1998).

The principal virulence factor that has been described for *K. pneumoniae* is its polysaccharide capsule. This capsular lipopolysaccharide appears in about 78 antigenic varieties based on classical serotyping. However, current classification based on capsular locus on the genome of these bacteria revealed more than 150 capsular locus (KL). Capsular locus is responsible for its mucoid colony phenotype. Some capsule types such as KL1 and KL2 were reported to be

more important than others. These unique capsular types mainly KL1 and KL2 are associated to hypervirulent strains. More detailed studies have revealed that capsular types are not the only factors defining hypervirulence property. There are also reports linking hypervirulent strains to the presence of plasmid-mediated regulators of mucoid phenotype (*rmpA/rmpA2*) genetic determinants. Other epidemic strains of *K. pneumoniae* belonging to other capsular types were also reported (Marr and Russo, 2019).

All strains of *K. pneumoniae* are resistant to ampicillin because of the presence of a chromosomal gene encoding a penicillin specific β-lactamase. In addition to inherent resistance to penicillins, nosocomial isolates have acquired other resistant genes to numerous other antibiotics. The increased antibiotic resistance of the nosocomial isolates might be because of the acquisition of mobile genetic elements or multidrug resistance plasmids. For example, *K. pneumoniae* is one of the most common organisms to carry plasmids encoding extended spectrum β-lactamases and bacteremia, and such strains are associated to higher rates of treatment failure and death (Spanu *et al*., 2006).

Genus *Enterobacter*: *Enterobacter* is another genus in the family *Enterobacteriaceae*, a Gram-negative, facultative anaerobic, rod-shaped, non-spore-forming bacterium widely encountered in nature. Members of the *E. cloacae* complex are clinically important as opportunistic bacteria and have emerged as nosocomial pathogens. *Enterobacter cloacae* complex shows further genomic heterogeneity (Davin-Regli and Pagès, 2015).

Based on biochemical and molecular studies, *E. cloacae* complex is classified in to six species: *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii* and *Enterobacter nimipressuralis*. *E. cloacae* and *E. hormaechei* are the most frequently isolated in human clinical specimens (Davin-Regli and Pagès, 2015; Peirano *et al*., 2018).

Though *Enterobacter spp.* are closely related to *Klebsiella spp*. phenotypic identification is difficult and not always reliable. Therefore, MALDI-TOF or molecular methods are reliable tools for the diagnosis of these bacilli. Strains of *E. cloacae* complex are most common causes of *Enterobacter spp.* related nosocomial bloodstream infections, but virulence-associated properties of these species are less understood.

These species are capable developing resistance to β-lactams through hyperproducing AmpC β-lactamases. These enzymes will be produced either by de-repression of a chromosomal bla_{AmpC} gene or by the acquisition of a transferable bla_{AmpC} gene on plasmids conferring antibiotic resistance (Mezzatesta *et al*., 2012). Other resistance phenotypes can be acquired that further increasing the resistance profile of the bacilli. Recent studies show that acquired resistance genes reduced effectiveness of most antibiotic families. However, most studies on antimicrobial susceptibility are focused on *E. cloacae*, *E. hormaechei* and *E. asburiae*. Similar to other *Enterobacteriaceae,* these strains produce ESBLs, carbapenemases and other resistance mechanisms to fluoroquinolones as well (Peirano *et al*., 2018).

Genus *Salmonella*: *Salmonella* is a Gram-negative, non-spore forming, facultative anaerobic bacilli that measure 2-3 µm in length and 0.4-0.6 µm in diameter that belongs to the *Enterobacteriaceae*. Like other members of the family, *Salmonella* produce acid on glucose fermentation, reduce nitrates, and do not produce cytochrome oxidase. All organisms are motile with peritrichous flagella, and most do not ferment lactose (Donnenberg, 2009).
Previously existence of multiple *Salmonella* spp. was taxonomically accepted, but now as a result of experiments indicating a high degree of DNA similarity, the genus *Salmonella* is classified into two species (Brenner *et al*., 2000). *Salmonella enterica*, contains six subspecies (I, II, IIIa, IIIb, IV, and VI), and *Salmonella bongori*, formerly subspecies V (Brenner *et al*., 2000; Desai *et al*., 2013). Except for rare infections with subspecies IIIa and IIIb serovars (formerly designated as genus *Arizonae); S. enterica* subspecies I contain almost all the serotypes pathogenic for humans. Members of the seven *Salmonella* subspecies can be serotyped into one of more than 2500 serotypes (serovars) according to antigenically diverse surface structures somatic (O) antigens, the carbohydrate component of lipopolysaccharide, and flagellar (H) antigens (Desai *et al*., 2013; Miller, 2009).

The etiologic agents of enteric fever *Salmonella* Typhi and *S.* Paratyphi serotypes (A, B, and C) were not reported to have hosts other than humans. Most commonly, foodborne or water borne transmission occurs because of fecal contamination by ill or asymptomatic chronic carriers. Usually, waterborne transmission involves the ingestion of fewer microorganisms and, as a result, has a longer incubation period and lower attack rate compared with food borne transmission. Although direct person to person transmission is not common, *S.* Typhi can be

transmitted sexually(Reller *et al*., 2003). Healthcare workers can acquire the disease from infected patients because of poor hand hygiene or handling laboratory specimens. Despite concerted effort against *Salmonella,* and most importantly typhoid fever, enteric fever continues to be one of global health problems (Miller, 2009).

Genus *Shigella*: *Shigella* is a non-motile, facultative anaerobe, Gram-negative rod. It is a member of the family *Enterobacteriaceae*. There are four species of *Shigella*: *S. dysenteriae* (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D). Groups A, B, and C cannot be distinguished biochemically; *S. sonnei* can be differentiated from the other serogroups by the expression of ornithine decarboxylase (DuPont, 2009).

Globally, Shigella is the second-leading cause of diarrheal disease after rotavirus. It is responsible for roughly 165,000 annual deaths worldwide, 12.5% of all diarrheal deaths. Most deaths occur in south Asia and sub-Saharan African countries. Mortality rates reduced significantly from previous decades largely because of improved sanitation, use of antibiotics, oral rehydration therapy (Kotloff *et al*., 2017). The transmission of *Shigella* is mainly through a direct spread from person to person or from contaminated food and water. Since intermediate bacterial replication is not required to achieve the low infectious dose, minimal infectious dose can be enough to transmit directly from contaminated fingers. In most cases transmission is by fecal-oral spread from people with symptomatic infection in developed countries. However, in low-income countries, both fecal-oral spread and contamination of common food and water sources remain an important mechanisms of transmission (DuPont, 2009). Pathologically, biochemically and genetically *Shigella spp*. are very similar to EIEC strains, and might have shared several genetic determinants (Croxen *et al*., 2013). The prototype diarrheal disease caused by *Shigella spp*. is shigella dysentery, a serious diarrhea, caused by invasive infection of the colon resulting in dysentery, characterized by diarrhea containing mucus, pus, and blood.

Shigella organisms can survive and transit through the stomach since they are less susceptible to acid than other bacteria; for this reason, as few as 10 to 100 organisms can cause disease. Ingested bacteria pass into the small intestine where they multiply; large numbers of bacteria then pass into the colon, where they enter the colonic cells. Because of its relatively low infectious dose, *Shigella* transmission can occur via direct person to person spread, as well as

via contaminated food and water. Humans are the only known natural reservoir for *Shigella* and the disease (DuPont, 2009).

I. Family *Morganellaceae* (Genus *Proteus*, Genus *Providencia*, and Genus *Morganella*)

The genera *Proteus, Providencia*, and *Morganella* are related members of the family *Morganellaceae* that are lactose negative, motile and produce phenylalanine deaminase (Donnenberg, 2009). There are several species of the Genus *Proteus*, but *P. mirabilis* and *P. vulgaris* account for most of the clinical isolates. Members of this genus also produce H_2S, both strains produce urease, and *P. vulgaris* produce indole. These bacteria are capable of swarming motility as they differentiate from typical enterobacterial bacilli expressing fimbriae and flagella into highly elongated rods with thousands of flagella that translocate rapidly across the surface of agar plates (Coker *et al*., 2000; Sabbuba *et al*., 2003). *Providencia stuartii* is the most common species of its genus isolated from clinical specimens, but *Providencia rettgerii* occasionally can be encountered. These bacteria can be differentiated from *Proteus* and *Morganella* based on their ability to use citrate and ferment D-mannitol. *Morganella morganii* is at present the only member of its genus and it is citrate negative (Donnenberg, 2009).

Proteus spp. are common causes of UTIs, occasionally in normal hosts and commonly in those with indwelling catheters or anatomic or functional abnormalities of the urinary tract. UTIs caused by *Proteus* spp. reported to be more severe than UTI caused by *E. coli* strains, with a higher proportion representing pyelonephritis. *Proteus spp*. are commonly isolated from the blood stream infections, the vast majority secondary to UTI, often associated with urinary catheters (Coker *et al*., 2000). *P. mirabilis* may also cause complicated urinary tract infection and bacteremia from a urinary tract infection. In addition to UTI, *Proteus spp*. can cause and frequently reported from other infections as well. These infections are higher particularly in hospitalized patients. It is reported that some strains of *P. mirabilis* can also cause diarrhea (Sabbuba *et al*., 2003).

II. Non-fermentative Gram-negative bacilli

Like *Enterobacterales*, non-fermenting Gram-negative rods are also a cause of significant morbidity and mortality. Non-fermeters are also commonly isolated from clinical specimens at

clinical microbiology laboratory. Species of main concern are *Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia,* and less frequently members of the *Burkholderia cepacia* group. *P. aeruginosa* is non-fermenting Gram-negative, motile, oxidase positive bacteria found in water, soil, and decomposing vegetation. They are generally considered saprophytic, however, these bacteria were frequently reported from opportunistic infections and very often from seriously ill hospitalized and immune-compromised patients (Donnenberg, 2009; Paterson, 2006b). Several studies have shown that they are associated with urinary tract infections, ventilator-associated pneumonia, cystic fibrosis, surgical site infections and bacteremia. *P. aeruginosa* and *A. baumannii* account for 10% of hospital acquired infections in US Intensive Care Units (ICUs) (Donnenberg MS, 2009).

Multidrug-resistance is common among non-fermenting Gram-negative rods of the clinically important *P. aeruginosa* and *A. baumannii* (Bassetti *et al*., 2018). These bacterial species possess a diversity of resistance mechanisms that lead to resistance against different classes of antimicrobials. Extended-spectrum β-lactamases (ESBLs) conferring resistance to extended-spectrum-cephalosporins, carbapenemases conferring resistance to carbapenems, and 16S rRNA methylases conferring resistance to all clinically available aminoglycosides are the most important causes of concern (Bassetti *et al*., 2018; Potron *et al*., 2015). Furthermore, concomitant resistance to fluoroquinolones, polymyxins (colistin) and tigecycline may lead to pandrug-resistance. It is evident that resistance to these drugs compromises treatment, leading to increased mortality, extended hospital stay and greater healthcare costs (Paterson, 2006b).

Trends of nosocomial *P. aeruginosa* infections show increasing antimicrobial resistance/MDR including carbapenems. Mechanisms of antimicrobial resistance include multidrug efflux pumps, β-lactamases and down-regulation of outer membrane porins (Hauser and Ozer, 2011). Biofilm formation and secretion of toxins are common mechanisms of virulence in *P. aeruginosa*. Effective management of infections caused by *P. aeruginosa* should consider prevention at source when possible and enhance control measures as necessary and prompt administration of proper antibacterial agents (Bassetti *et al*., 2018). Multidrug-resistant *P. aeruginosa* may require treatment with less commonly used antimicrobial agents (Hauser and Ozer, 2011).

Acinetobacter baumannii is another non-fermenting Gram-negative bacillus, non-motile, obligate aerobic coccobacillus harboring many virulence factors. The ability to attach and persist on solid and dry surfaces, to obtain essential nutrients such as iron, to adhere and subsequently destroy epithelial cells, and the ability in some strains to produce gelatinases and proteinases to damage host tissues matrixes (Camp and Tatum, 2010). *A. baumannii* species commonly cause nosocomial infections, predominantly aspiration pneumonia, ventilator-associated pneumonia and catheter-associated bacteremia, but can also cause soft tissue and urinary tract infections (Wong *et al*., 2017). Other *Acinetobacter* species including: *A. pittii*, *A. noscomialis*, *A. seyferti* and *A. dijkshornii* were also reported to cause bacteremia and nosocomial infections (Chusri *et al*., 2014; Cosgaya *et al*., 2016; Wong *et al*., 2017).

A. baumannii is clinically important and increasingly apparent for its antimicrobial resistance property. It rapidly developed resistance to β-lactams, carbapenems and other classes of drugs. It has the capacity to swiftly change and adapt to a selective environment (Peleg *et al*., 2008). *A. baumannii* has an ability to asymptomatically colonize both skin of patients and healthy individuals. The transmission of colonizing bacteria to a susceptible patient, however, can result in infection. *A. baumannii* also has the ability to form biofilms, which may play a role in the process of colonization (Chebotar *et al*., 2014).

In general, Gram-negative bacilli, both carbohydrate fermenters and non-fermenters are a significant problem of public health. Gram-negative bacilli are known causes of both nosocomial and community acquired infections. These bacilli are ubiquitous in the environment. The spread of the bacteria with acquired/modified property with antimicrobial resistance and virulence profile is a clear challenge to clinical care and public health. Scientific approaches addressing the question of development of antimicrobial resistance, dissemination of resistance strains, and containment of these strains is extremely important. With the current pace of antimicrobial resistance development to available antimicrobials and lack of enough advances in new drug discovery, the future seems challenging for vulnerable people in the population. Surveillance strategies, and surveillance networks should be instituted from local single institution, national to international agencies to put together efforts, resources, and knowledge to control the problem.

1.2.3 β-lactam antimicrobials

β-lactams are a large group of antibiotics that inhibit growth of bacteria by inhibiting synthesis of cell wall of bacteria. These groups of antibacterials can be classified into four major chemical groups: penicillins, cephalosporins, carbapenems, and monobactams (Rahman *et al*., 2018). The extensive usage of these compounds in both humans and animals is due to their excellent safety and broad-spectrum activity. The generation of several subgroups of the antimicrobials by modification of the basic β-lactam compound further increased the diversity and spectrum of activity of the new generation of antimicrobials.

a. Structure of β-lactam antimicrobials:

The structure of β-lactam antimicrobials has conserved β-lactam ring in common. Structurally β-lactam ring consisting of three carbon atoms and one nitrogen atom. The β-lactam structure is linked to thiazolidine ring through nitrogen atom. In penicillins, the N-acyl attachment determines the activity and spectrum of the 6-amino-penicillanic acid. In cephalosporins, the β-lactam ring and dihydrothiazine ring are merged, whereas, in carbapenemes, the β-lactam ring is joined with a hydroxyethyl side chain deficient of an oxygen or sulfur atom in the bicyclic nucleus. Monobactams have no additional ring structure (Bush and Bradford, 2016; Rahman *et al*., 2018) (**Figure 1.1).**

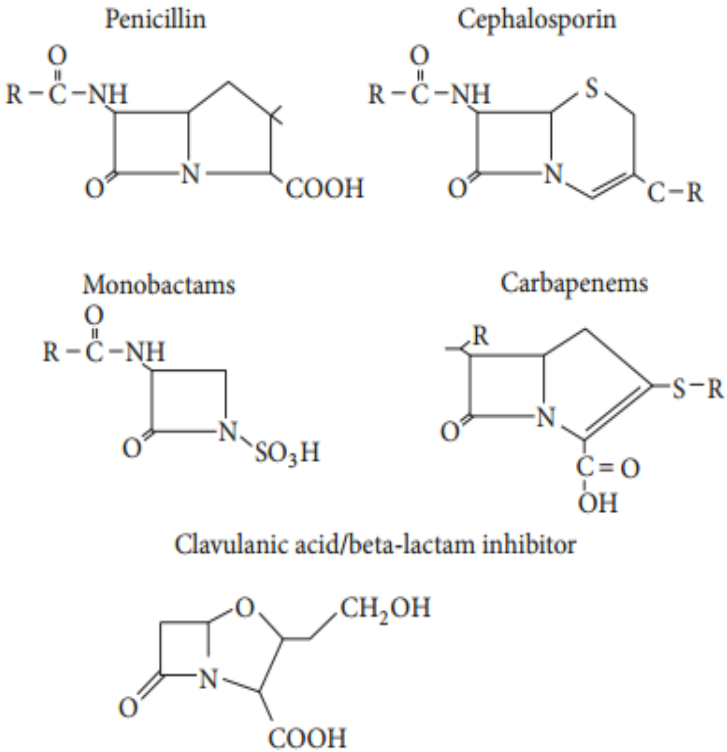

Figure 1.1: Basic structure of β-lactam antimicrobials (Rahman *et al*., 2018)

b. Mechanisms of action of β-lactam antimicrobials:

The β-lactams mechanism of action is through binding to enzymes that are used for synthesis the cell wall called peptidoglycan, an important structural constituent of bacterial cell survival. These enzymes include carboxypeptidases and transpeptidases used for synthesis of cell wall. The PBPs catalyze cross-linking of amino acids to chains of adjacent amino acids in to a mesh in a periplasmic space. The β-lactam ring is similar to the D-alanine-D- alanine of the N-acetyl muramic acid (NAM) pentapeptide chains (Karen and Jacoby, 2010; Rahman *et al*., 2018).

Because of structural similarity between the D-alanine-D-alanine and the β-lactam ring, PBPs pick the β-lactams for the synthesis of cell wall peptidoglycan. This process leads to acylation of the PBPs that in turn impede or block the enzyme activity.

Once the enzyme is inactive the transpeptidation reaction is inhibited, the accumulation of cell wall precursors induces the activation of cell wall autocatalytic systems. Hence, β-lactams eliminate bacteria by simultaneously blocking transpeptidases and activation of autolysins (Isturiz, 2008; Bush *et al*., 1995; Livermore, 2008).

c. β-lactamases, and ESBLs

Resistance to β-lactams has probably arisen throughout bacterial history but has become a useful and therefore selected trait since the β-lactam antibiotics came into clinical use. These drugs exerted evolutionary selection, killing susceptible bacteria and allowing the resistant ones to survive (Bradford, 2001). Resistance to β-lactams may be inherent to a species, as seen in *Enterococci*, which lack PBPs that are required to bind cephalosporins. It may be acquired through spontaneous mutation or DNA transfer. Functionally, β-lactam resistance may be a result of the production of β-lactamases, impermeability, efflux, and target modification. The resistance mechanisms may occur singly or together with other mechanism (Thirapanmethee, 2012; Karen and Jacoby, 2010).

Changes in the normal PBPs or acquisition of other β-lactam insensitive PBPs are most common mechanisms of resistance in Gram-positive cocci (pneumococci and MRSA). However, in Gram-negative bacteria, resistance is mostly due to a combination of either endogenous or acquired β-lactamases, along with naturally up regulated impermeability and efflux systems (Dzidic *et al*., 2008). β-lactamases are enzymes that can inactivate β-lactam antimicrobials by hydrolyzing the β-lactam ring.

There are different genetic variants of β-lactamases or isoforms of the same genetic variants that differ in few amino acids. These enzymes differ by few amino acids at least at one or more positions, and this difference in at least one amino acid significantly increased the affinity and spectrum of activity of the enzymes to several of β-lactam substrates. A scheme of functional classification of these enzymes partly uses the affinity and spectrum activity of these enzymes to classes/generations or sub-generation of penicillin's and cephalosporins and other properties to β-lactam inhibitors (Liebana *et al*., 2013) (**Table:1.1).**

One of such group of β-lactamases are extended-spectrum β-lactamases (ESBLs). Though, there were no consensus on the precise definition of ESBL, a commonly used working

definition is that ESBLs are β-lactamases capable of conferring bacterial resistance to the penicillins; first-, second-, and third-generation cephalosporins; and aztreonam (but not the cephamycins and carbapenems) by hydrolysis of these antibiotics, and they are inhibited by β-lactamase inhibitors such as clavulanic acid (**Table 1.1**).

Table 1.1: Main hydrolytic characteristics of ESBLs and AmpC β-lactactamases (Liebana *et al*., 2013)

Antimicrobial classes	Examples of antimicrobials	ESBL hydrolytic activity	AmpC hydrolytic activity
Penicillins	Bensylpenicillin, ampicillin, amoxicillin, ticarcillin, piperacillin	+++	+++ (exception is ticarcillin if produced only at basal level)
Penicillin/β-lactam inhibitor	Amoxicillin-clavulanic acid, ampicillin-sulbactam, piperacillin-tazobactam	Inhibitor usually neutralizes ESBL activity	AmpC activity is not neutralized by inhibitor
First-gen-cephalosporin	Cefazolin, cefalexin, cephalothin	+++	+++
Second-gen-cephalosporin	Cefaclor, cefadroxil, cefuroxime	++	++
Third-gen-cephalosporin	Ceftriaxone, cefotaxime, ceftazidime	++	- (if produced at basal level) +++ (if over produced)
Fourth-gen-cephalosporin	Cefepime, cefpirome	++	+/-
Monobactams	Aztreonam	++	++
Cephamycins	Cefoxitin, cefotetan	-	+++
Carbapenems	Imipenem, meropenem, ertapenem	-	- or+/- (depending on the expression level)

Abbreviations: +++, high level hydrolysis: ++, medium level hydrolysis; + , weak level of hydrolysis; +/-, none or weak hydrolysis which can continue to and influence susceptibility results usually if combined with other resistance mechanisms; -, no hydrolysis;

1.2.4 Classifications and Families of β-lactamases

Developing a standardized classification of β-lactamases and ESBLs were challenging until this date. Several of the classifications attempted and published to simplify classification and improve understanding about ESBLs have their own advantages and disadvantages. None of the attempts have answered the simplicity required for the complexity of the nature of enzymes in relation to the functional and genetic diversity to clinical and epidemiologic usability. The Ambler molecular classification and the Bush-Jacoby-Medeiros functional classification are the two most commonly used classification systems in β-lactamases (Bebrone, 2007; Heritage *et al*., 1999; Karen and Jacoby, 2010). Based on Ambler classification β-lactamases classified in to four major classes (designated Class A, -B, -C to D). The basis of this classification

scheme is an amino acid sequence similarity in the proteins/enzymes (Hall and Barlow, 2005). Class A, class C, and class D) are serine proteins (β-lactamase enzymes that have serine in the catalytic domain). Whereas, class B enzymes are metallo-β-lactamases that contain a metal ($Zn^{+2)}$ in the catalytic domain of these group of enzymes. All ESBLs, with the exception of OXA-type enzymes (which are class D enzymes) belong to molecular class A (Hall and Barlow, 2005).

The Bush-Jacoby-Medeiros classification scheme groups β-lactamases according to functional similarities, substrate and inhibitor profile (Karen and Jacoby, 2010). Considering this classification system, there are three main groups and multiple subgroups of β-lactamases. The functional classification has a better and immediate relevance to the physicians in clinical care and/or for a clinical microbiologist in diagnostic laboratory. This method details the actual interaction between β-lactamase and β-lactam substrates that are clinically apparent (Paterson *et al*., 2005). According to the functional classification, ESBLs belong to group 2be or group 2d. The 2d (bla_{OXA}) types share most of the basic features of group 2be enzymes, but they are different that the bla_{OXA} types are β-lactamase inhibitor resistant.

The 2be notation of the functional classification describes that these enzymes are derived from group 2b β-lactamases like bla_{TEM-1}, bla_{TEM-2} and bla_{SHV-1} and the 'e' in 2be denotes that these β-lactamases have an extended spectrum of activity (**Table 1.2**). The ESBLs derived from bla_{TEM-1}, bla_{TEM-2} or bla_{SHV-1} differs from their progenitors by as few as one amino acid. The change/mutation in at least as few as one amino acid position may result in a profound change in the enzymatic activity of the ESBLs. They can now hydrolyze the third generation cephalosporins or aztreonam (extension of spectrum compared to the parent enzymes) (Paterson *et al*., 2005; Karen and Jacoby, 2010). Inhibition by β-lactamase inhibitors such as clavulanic acid and inability to hydrolyze cephamycins differentiates ESBLs from AmpC-type β-lactamases, which have third-generation cephalosporins as their substrates but which are not inhibited by clavulanic acid (Bush *et al*., 1995). Selection of stably depressed mutants which hyperproduce the AmpC-type β-lactamases has been associated with clinical failure when third generation cephalosporins are used to treat serious infections with organisms producing these enzymes (Jacoby and Munoz-Price, 2005; Padmavathy *et al*., 2013).

The Bush-Jacoby-Medeiros and Ambler's classifications, both have their own advantages and disadvantages. But, because Bush-Jacoby-Medeiros classification is a functional classification, it can be easily used for guiding treatment for clinicians. Combination of hydrolytic activity of the β-lactamases, spectrum of substrates, and susceptibility of the β-lactamases to β-lactamase inhibitors are major factors considered for classification in Bush-Jacoby-Medeiros functional classification.

Ambler's molecular classification can give details of genetics and understanding of the enzymes' proteomics. However, it is not easy to translate the Ambler's classification into immediate clinical use. There are several reviews and studies that successfully put the two classification methods together for comprehensive understanding of the classification and generation of β-lactamases. The fundamental bases for both classification schemes were used to increase comprehension, so that epidemiologists, scientists, students, and clinicians may use it. However, all the classifications attemted so far do not seem to have met the simplicity and comrehensive underetsanding easier for all parties including policy makers and the public too **(Table 1.2).**

Table 1.2: Classification of β-lactamases based on Functional and Molecular features (Bush and Jacoby, 2010)

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros Group (1995)	Molecular class (sub-class)	Distinctive substrate(s)	Inhibited by		Defining characteristics	Representative enzyme(s)
				CA or TZB[a]	EDTA		
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillins; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FoX-1, MIR-1
1e	NI[b]	C	Cephalosporin	No	No	Increased hydrolysis of ceftazidime and often other oxyimino β-lactams	GC-1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	2b	A	Penicillins and early cephalosporins	Yes	No	Similar hydrolysis for benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended spectrum cephalosporins	Yes	No	Increased hydrolysis of oxyimino-β-lactams (ceftriaxone, cefotaxime, ceftazidime, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillin	No	No	Resistance to clavulanic acid, sulbactam, tazobactam	TEM-30, SHV-10
2ber	NI	A	Extended spectrum cephalosporins and monobactams	No	No	Increased hydrolysis of oxyimino β-lactams combined with resistance to clavulanic acid, sulbactam and tazobactam	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, cefpirome	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	NI	D	Extended spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino β-lactams	OXA-11, OX-15
2df	NI	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenemes	OXA-23, OXA-48
2e	2e	A	Extended spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not by aztreonam	CepA
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	3	B(B1)	Carbapenems	No	Yes	Broad spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1,
		B(B3)					L1, CAU-1, GOB-1, FEZ-1
3b	3	B(B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
NI	4	Unknown					

[a]CA - clavulanic acid, TZB- tazobactam, [b]NI- not included.

1.2.5 Mechanisms of Resistance in ESBL-producing Bacteria

Bacteria can resist antibiotics as a result of biochemical alteration or genetic adaptability that can be achieved either by chromosomal mutation or by exchange of genetic materials (Dzidic *et al*., 2008). These mechanisms of resistance to antimicrobial agents can be due to (i) impermeability of the drug: this is the most frequent cause of intrinsic resistance. Resistance in *P. aeruginosa* is an example of such mechanisms; (ii) alteration in target molecules: in the case of methicillin resistant *S. aureus* with altered penicillin binding proteins; (iii) enzymatic drug modifications: as in β-lactamase enzymes hydrolytic activity on penicillins and cephalosporins. These β-lactamases affect a common structure in a drug molecule, β-lactam ring. Penicillins, cephalosporins, monobactams and carbapenems can all be hydrolyzed by multiple members of the β-lactamase family of enzymes, resulting in ineffective compound. The other important classes of antibiotics are aminoglycosides, which are destroyed by the action of aminoglycoside modifying enzymes produced by the bacteria; (iv) efflux: the role of efflux of drug from the bacterial cell as a resistance mechanism is common among clinically important strains (Tenover *et al*., 1999; Dzidic *et al*., 2008; Sun *et al*., 2014) (**Figure 1.2)**.

Although both chromosomal mutations and genetic transfer can be responsible for the resistance acquisition, it is the transferable resistance which poses a great threat as it can achieve much larger dimensions due to wide and rapid dissemination. This transferable resistance is carried on R-plasmids (Feizabadi *et al*., 2010; Dzidic *et al*., 2008), a single plasmid can carry a number of genes coding for multiple drug resistance (Sturm *et al*., 2010). Subsequently, in the presence of favorable genetic environment, antimicrobial therapy leads to pervasive changes in the structure of microbial community. Persistence may increase among some strains in the presence of antibiotic, and resistance among other strains. Thus, resistant strains will improve their survival advantage over other strains of the same species and/or other species. This leads to rapid proliferation and persistence of antimicrobial resistant strains initially present in low basal levels in microbial ecosystem, and also these resistant strains disseminate the genetic determinants of antimicrobial resistance through both horizontal and vertical genetic transfer (Peleg and Hooper, 2010).

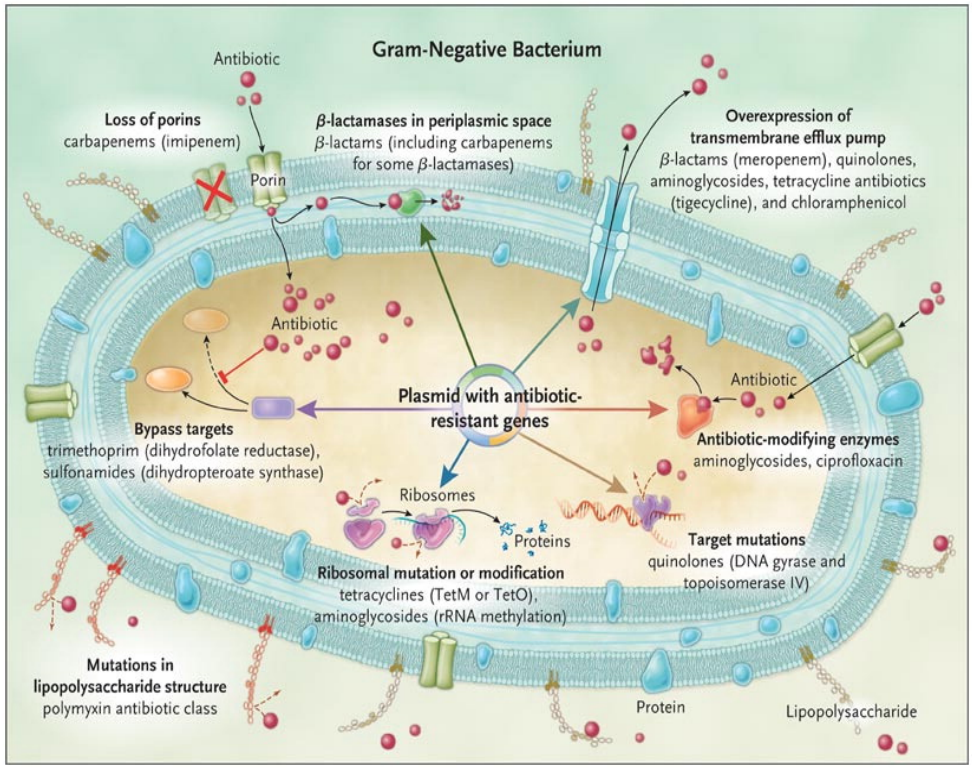

Figure 1.2: Mechanisms of resistance in Gram negative bacteria (Peleg and Hooper, 2010)

1.2.6 Genetic determinants of ESBLs

Genetic determinants of β-lactamases and/or ESBLs show huge variety of distinct hydrolytic proteins/enzymes to β-lactam antimicrobials. These enzymes are chromosomally encoded genes or genes acquired on mobile genetic elements. Acquired genes could be integrated in to chromosomal structures or can be carried on plasmids. Factors responsible for faster dissemination and spread of ESBL is the horizontal transfer of mobile genetic elements carrying factors for ESBL enzymes occurs either through horizontal transfer of genes, mediated by mobile genetic elements (MGE) or by clonal expansion of resistant strains (Foley *et al*., 2009).

Mobile genetic elements are segments of DNA that encode enzymes and other proteins that mediate movement of DNA with in genomes (intracellular mobility) or between bacterial cells

(intercellular mobility) through either conjugation, transformation or transduction (Bellanger *et al*., 2014). Types of MGE fall into two general types; elements that can move from one bacterial cell to another, which in terms of antibiotic resistance includes resistance plasmids, conjugative resistance transposons, and bacteriophages and elements that can move from one genetic location to another in the same cell (Frost *et al*., 2005). Plasmids and conjugative transposons transfer from one cell to another by mechanisms that involve replication (Bennett, 2008). Transposons, gene cassettes and *IS*CR mediated gene transfer between sites on the same or on different DNA molecules require some form of recombination, which may or may not include replication. Plasmids accumulate antibiotic resistance genes as a consequence of the activities of at least three recombination systems (Bennett, 2008).

a. Plasmids and conjugative elements

Plasmids are extra-chromosomal segments of DNA. Classic plasmids are covalently closed, circular, double-stranded DNA, much smaller than bacterial chromosome. Plasmids larger than 20 kb are often conjugative and can promote their own transfer between bacterial hosts (Frost *et al*., 2005). Plasmid complexity increases with size and larger plasmids can be as big as the size of smaller bacterial chromosome and may contain several co-integrated compatible replicons. Resistance plasmids carry resistance genes often organized into integrons or carried on transposons. Other plasmids carry metabolic genes or act as sex factors to promote transfer of the bacterial chromosome (Bennett, 2008). Horizontal genetic transfer through plasmids plays a major role for the constantly evolving bacterial antimicrobial resistance.

Currently, there are many plasmids defined as responsible for antimicrobial resistance in bacteria classified as *Enterobacterales* (Carattoli, 2009). Based on PBRT (PCR-based replicon typing) assay, about 28 different plasmid (replicon) types were identified to be associated with *Enterobacterales.* Some plasmid families are largely prevalent among *Enterobacteriaceae* and also associated with specific resistance genes (Lynch *et al*., 2013). Some of these plasmids have a broad range of hosts and are able to transfer antimicrobial resistance and virulence factors to different species or classes of bacteria, and moreover, these plasmids were detected in different countries and in bacteria of different hosts and sources (Carattoli, 2013). Such plasmids can be considered main genetic factor for spreading antimicrobial resistance. The

detection of these types of plasmids seems tightly linked to positive selection exerted by antimicrobial use. An increased prevalence of particular plasmids compared to those observed in a given bacterial population that were not preselected with certain antimicrobials may have epidemiologic importance. However, *IncF* family of plasmids are naturally common in fecal microbiota of humans and animals, regardless of resistance genes (Carattoli, 2013; Lynch *et al*., 2013).

b. Integron and gene cassettes

Integrons are genetic elements able to acquire and rearrange open reading frames (ORFs) embedded in cassettes. Integrons can convert these genes to functional genes and ensure correct expression to corresponding polypeptides (Tennstedt *et al*., 2003). The ability of integrons to capture multiple exogeneous genes enabled them to carry multiple antimicrobial resistance genes. Class I and class II integrons were associated to carriage of multiple antimicrobial genes (Lin *et al*., 2016). Multiple antibiotic resistances are strongly associated with integrons. The natural recombination systems that can be transferred in companion with mobile genetic elements play a major role in spreading antibiotic resistance genes in clinical isolates.

Nine classes of integrons have been identified based on nucleotide sequence of the integrase gene. Their basic structure consists of three core features: i) an integrase encoding gene (*intI*), ii) recombination site (*attI*), and iii) an integrin associated promoter (*Pc*). Acquisition of new genes on integrons is like gene acquisition of new genes on gene-cassettes. Gene cassettes are also simple structures consisting of single open reading frame (ORFs) and cassette associated element, historically called a 59 base element, referred to as *attC* (Cury *et al*., 2016).

Class I and class II integrons have a similar organization. The class II integrase has 46% amino acid identity to class I integrase (Partridge, 2011). Integrons are versatile and efficient gene capturing and expressing elements commonly found in genomes of bacteria and reported in different studies as playing an important role in dissemination of antimicrobial resistance genes.

The gene capture systems in integrons utilize site specific recombination, not transposition mechanisms. Integrons have recombination system which consist of a gene called *int* that in turn encodes a site specific recombination enzyme (integrase) and a site at which short DNA sequences captured are inserted by the integrase (Gillings, 2014), and gene cassettes move

from one integron to another or from one site on an integron to another on the same integron. A gene cassette exists as a small autonomous non-replicating double stranded circular DNA molecule. This state is an intermediate in the mechanism that mediates cassette transfer from one integron to another or the re-assortment of gene cassettes within a particular integron (Li *et al*., 2013; Partridge, 2011).

c. Transposons

Transposon is mobile unit of DNA that can jump/transpose from one DNA molecule to another, or may jump from a plasmid to a chromosome or from plasmid to plasmid. Mostly without site specific transposition (Frost *et al*., 2005). Transposable elements possess two main characteristics that differentiate them from other genetic elements. As they are mobile, they are capable of changing their genetic environment and in doing so they alter the genetic environment of the locus into which they insert (Iyer *et al*., 2013).

Transposons belong to a set of mobile elements/transposable elements that includes a small cryptic element called insertion sequences (IS elements). Transposon encodes at least one function that changes the phenotype of the cell, a resistance transposon confers resistance to a particular antibiotic(s), whereas insertion sequence elements do not encode for a protein that can influence physiology of bacteria (Frost *et al*., 2005).

The generation of types and the quest for standard classification of transposons is growing with the availability of large whole genome data. In a classical classification, classified broadly in to class I-transposons and class II-transposons. Class I transposons are called retro-transposons (replicate through RNA intermediate for transposition) has a pair of insertion sequences that flank the resistance genes. Class II-transposons (DNA transposons) do not require RNA intermediate.

Transposons may carry terminal inverted repeat segments to enclose the genes, transposase (*tnpA*), a resolvase (*tnpR*), and one or more antibiotic resistance genes. Some transposons are conjugative (Frost *et al*., 2005; Partridge, 2011).

Gram-negative bacilli, the *Enterobacterales*, are adapted to exchanging genetic information and antibiotic resistance traits. These traits (genes) are not intrinsically mobile and appear to have been captured from the chromosomes of various species, where they may have originally

had other functions (Paterson, 2006a). Such capture involves two different types of mobile genetic elements: (i) those able to transfer genes between DNA molecules or mobile elements (for example: insertion sequences (IS), gene cassettes, integrons, and transposons and (ii) those able to transfer between cells (for example: conjugative and mobilizable plasmids and integrated conjugative elements) (Partridge, 2011; Dzidic *et al*., 2008).

d. Bacteriophages

Bacteriophages are bacterial viruses which also use transposition to replicate and may encode for several functional proteins including toxins and antimicrobial resistance, but only the temperate phages can integrate into bacterial genome and bear the risk. On the contrary, recent advances in phage related studies are targeting lytic phages as an alternative therapy to drug resistant strains, and preliminary evidence could suggest that this is possible (Hill *et al*., 2018). Phages can carry antimicrobial resistance genes and disseminate resistance including ESBLs, and fluoroquinolone resistance genes especially among bacteria that cause food borne diseases (Brown-Jaque *et al*., 2018; Colavecchio *et al*., 2017). Therefore, the extent of phage-mediated dissemination of antimicrobial resistance should be carefully elucidated.

Generally, β-lactamase genes encoding ESBLs have been reported in *Enterobacterales* and were associated with mobile genetic elements (MGEs), such as insertion sequences (ISs), integrons, transposons, plasmids and phage-related elements (Frost *et al*., 2005). The diversity and continuous evolution of these mobile elements or genetic vehicles is responsible for evolution and spread of genes encoding for ESBLs and subsequently increased population of ESBL-producing Gram-negative bacteria in both humans and animals (Carattoli, 2009; Piroth *et al*., 1998).

1.2.7 Clonal expansion of resistant strains

Clonal expansion refers to a particular bacterial cell line multiplying and disseminating in a community or causing an outbreak in a hospital (Lopez-Causape *et al*., 2013; Matsumura *et al*., 2013). The bacteria are then spread vertically multiplying through cell division and may disseminate in a population including patient population, animals, food and environment (Klugman, 2003).

In a continental surveillance study in Europe, a high prevalence of ESBL gene was detected and spread of the resistance genes was caused by dissemination of epidemic plasmids through both clonally related and clonally unrelated species (Canton *et al*., 2008). Mainly, *K. pneumoniae* and *E. coli* were responsible for most of both community and nosocomial acquired infections. The role of clonal strains to carry epidemic plasmids were also equally important for the dissemination of several of the well characterized ESBL-genes, bla_{TEM} ($bla_{\text{TEM-24}}$, $bla_{\text{TEM-4}}$, $bla_{\text{TEM-52}}$), bla_{SHV} ($bla_{\text{SHV-5}}$, $bla_{\text{SHV-12}}$) and $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-14}}$ or $bla_{\text{CTX-M-15}}$) (Canton *et al*., 2008). It has become an established fact now that dissemination of antimicrobial resistance through both local and international clones like *E. coli* (ST131) and *K. pneumoniae* (CC258) is an important milestone for sentinel and surveillance strategies. These clones remain a concrete evidence for global dissemination of antimicrobial resistance through specific lines of clones. Several other studies also indicated that clonal spread as one of the major factors responsible for an increase in the current prevalence and global dissemination of ESBL-producing *Enterobacteriaceae* (Izdebski *et al*., 2013; Khattak and Fraise, 2011; Coque *et al*., 2008; Tschudin-Sutter *et al*., 2012).

Another study from Taiwan, revealed that an increase in rate of ESBL-producing isolates in *E. coli* and *K. pneumoniae* species might have been due to the horizontal transfer of resistance plasmids, which are primarily from clonal expansion of some epidemic clones of *K. pneumoniae* and further disseminated through strains of other clonal lineage and increased the dimension of dispersion of ESBL-producing strains (Lin *et al*., 2010).

Nationwide dissemination of particular multidrug resistant clones of *Enterobacteriaceae* has also been observed (Sader *et al*., 2014). There are a number of studies from Europe reporting that strains of the same clone causing an outbreak at different parts of the region. But from

Africa, a study in Madagascar revealed dissemination of ESBL-producing bacteria at one of the hospitals in Antananarivo was polyclonal, unrelated patterns of ERIC-PCR (Rakotonirina *et al*., 2013; Tansarli *et al*., 2014). Now, there are mechanisms of genetic basis for the evolution and spread of ESBL-producing *Enterobacteriaceae* and other Gram-negative bacteria. These mechanisms and the clonal strains may vary from one region of the world to the other. However, such data is lacking from low-income countries, and remains inconclusive, whenever molecular data is available in most cases the sample size is small.

1.2.8 Molecular evolution and diversity of ESBLs

β-lactamases may be chromosomally encoded and universally present in a species or plasmid coded and only present in some strains that acquired the plasmid (Chong *et al*., 2011). The chromosomal enzymes are believed to have evolved from PBPs with which they show same sequence homology, probably a result of selective pressure exerted by β-lactam producing soil organisms found in the environment (Poirel *et al*., 2012). Most of the ESBLs have evolved by genetic mutation from parent β-lactamases, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{TEM-2}}$, and *bla*$_{\text{SHV-1}}$. These parent enzymes are commonly found in Gram-negative bacteria mainly in *Enterobacteriaceae* (Rupp and Fey, 2003; Ramphal and Ambrose, 2006; Severin *et al*., 2012). However, the hydrolytic activity of these parent enzymes is less, and the spectrum activity is limited. Further modification/mutation of the parent enzymes at one or few amino acids changes the potency of hydrolytic activity and the spectrum of substrate they can inactivate and thus generates several variants of these enzymes. The *bla*$_{\text{CTX-M}}$ and GES are also a group of ESBLs that are divided in to several other variants of enzymes because of amino acid substitution. Unlike *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{SHV-1}}$, *bla*$_{\text{CTX-M}}$ parent enzymes are more reactive in their natural homologue, as first phase classified as *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{CTX-M-9}}$, and *bla*$_{\text{CTX-M-25}}$ with ≥90% amino acid similarity in these subgroups (Gniadkowski, 2008).

a. TEM-1

The first plasmid mediated β-lactamase in Gram-negative bacteria, *bla*$_{\text{TEM-1}}$ was described in the early 1960s. Plasmid and transposon mediated *bla*$_{\text{TEM-1}}$ enzymes spread worldwide and are now found in many different species of the family *Enterobacterales*, and other Gram-negative bacteria (Canton *et al*., 2008). More than 150 TEM-type β-lactamases have been found, and

all of them are derivatives of *bla*$_{TEM-1}$ or *bla*$_{TEM-2}$ by point mutations. The *bla*$_{TEM-1}$ was first demonstrated in 1965 in an *E. coli* isolate from a patient in Athens, Greece, named Temoneira (designation TEM).

In contrast to the majority of *bla*$_{TEM}$ β-lactamases, *bla*$_{TEM-1}$, *bla*$_{TEM-2}$ and *bla*$_{TEM-13}$ are not ESBLs and are only able to hydrolyze penicillin. Some *bla*$_{TEM}$ derivatives have been found to have a reduced affinity for β-lactamase inhibitors and are called Inhibitor Resistant TEM (IRT). These enzymes have negligible activity against extended-spectrum cephalosporins and are not considered to be ESBLs (Karen and Jacoby, 2010).

b. SHV-1

Another family of β-lactamases is the SHV enzymes (sulfhydryl variable type). A progenitor of SHV enzymes, *bla*$_{SHV-1}$ was first described in *K. pneumoniae*. *bla*$_{SHV-1}$ confers resistance to broad spectrum penicillins. In 1983, *Klebsiella ozaenae* strain was isolated in Germany possessing *bla*$_{SHV-2}$ enzyme that efficiently hydrolyzed cefotaxime and to a lesser extent ceftazidime. Currently more than 450 SHV-derivatives are known, all being derivatives of *bla*$_{SHV-1}$ or *bla*$_{SHV-2}$. Like *bla*$_{TEM}$-type enzymes majority of the *bla*$_{SHV}$ enzymes are ESBLs. Over the years, use of newer β-lactam antibiotics has enabled selection of new variants of β-lactamases (Heritage *et al*., 1999). After the introduction of third generation (oxyimino cephalosporins) in to clinical practice, prevalence and spread of the β-lactamases producing strains have been increased (Al-Agamy, 2013).

There are several groups of ESBLs with similar behavior but different evolutionary histories. The largest groups are the mutants of *bla*$_{TEM}$ and *bla*$_{SHV}$ β-lactamases. The mutations which affect a small number of critical amino acids enlarge the enzyme's active site and enable it to deflect the oxyimino substitutes, which normally shield the β-lactam ring. Classical *bla*$_{TEM}$ and *bla*$_{SHV}$ enzymes are unable to significantly hydrolyze the oxyimino-cephalosporins where the mutants can do so, conferring resistance to antimicrobials of extended spectrum than their host strains (Lewis *et al*., 2007; Naas *et al*., 2008)

c. CTX-M

A third family consists of the *bla*$_{CTX-M}$ enzymes, which are also ESBLs (Lewis *et al*., 2007). The designation of CTX-M reflects the hydrolytic activity of these β-lactamases activity to cefotaxime. The *bla*$_{CTX-M}$ enzymes have ≤40% amino acid identity with *bla*$_{TEM}$ and *bla*$_{SHV}$ type

ESBLs. More than 230 variants/mutant of *bla*$_{CTX-M}$ enzymes have been well recognized until March 2020(http://www.bldb.eu/BLDB.php?prot=A accessed on 2020/04/01). These enzymes are divided into five clusters on the basis of the amino acid sequence: *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-8}$, *bla*$_{CTX-M-9}$ and *bla*$_{CTX-M-25}$ (Lewis *et al*., 2007). Most of the subgroups have evolved from chromosomal β-lactamase genes escaping from *Kluyvera* spp., an enterobacterial genus of little clinical importance. Having migrated to mobile DNA, the *bla*$_{CTX-M}$ β-lactamases may have evolved into more diverse and several *bla*$_{CTX-M}$ ESBL-genes. A rapid diversification and increased prevalence of *bla*$_{CTX-M}$ enzymes in *Enterobacteriaceae*, and other Gram-negative bacilli caught the attention of clinicians', microbiologists' and researchers' during the early 2000s (Bradford, 2001; Bush *et al*., 1995; Naas *et al*., 2008).

It evolved from the first identified *bla*$_{CTX-M}$ which were able to confer cefotaxime resistance and very low/no capacities to efficiently hydrolyze ceftazidime to now more than 60% of *bla*$_{CTX-M}$ variants and able to confer cefotaxime and ceftazidime resistance simultaneously. And thus, ceftazidime might have been one of the main selective forces contributing to the diversification of *bla*$_{CTX-M}$ variants (Gniadkowski, 2008).

Recombination events might have also accelerated the evolution of *bla*$_{CTX-M}$ genes in at least some of the variants. A recombination requires the simultaneous presence of both *bla*$_{CTX-M}$ variants in a same microhabitat. A recombination event was described as one of the drivers of the generation of *bla*$_{CTX-M-64}$ enzyme. It was described that recombination between members of the *bla*$_{CTX-M-9}$ and *bla*$_{CTX-M-1}$ derived genes, probably *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$, the most prevalent variants might have evolved to a recombinant *bla*$_{CTX-M-64}$ variant (Cantón *et al*., 2012).

d. OXA

The *bla*$_{OXA}$-type β-lactamases are named-OXA because of their ability to hydrolyze oxacillin. They predominantly occur in *P. aeruginosa* but have been detected in many other Gram-negative bacteria (Livermore, 2008). The *bla*$_{OXA}$-type ESBLs were originally discovered in *P. aeruginosa* isolates. OXA-type ESBLs evolved from narrow spectrum parent enzymes much more similar with the evolution of *bla*$_{SHV}$ and *bla*$_{TEM}$ type ESBLs. The *bla*$_{OXA-10}$ (parent OXA-type ESBL), weakly hydrolyzes cefotaxime, ceftriaxone and aztreonam, giving most organisms reduced susceptibility to these antibiotics (Bradford, 2001). But through mutation

in the amino acid sequence, a new enzyme with extended spectrum (activity to ceftazidime) evolved to the first ESBL *bla*$_{OXA}$ type (*bla*$_{OXA-11}$). Whereas, *bla*$_{OXA-14}$, *bla*$_{OXA-16}$, *bla*$_{OXA-17}$, *bla*$_{OXA-19}$, *bla*$_{OXA-15}$, *bla*$_{OXA-18}$, *bla*$_{OXA-28}$, *bla*$_{OXA-31}$, *bla*$_{OXA-32}$, *bla*$_{OXA-35}$ and *bla*$_{OXA-45}$ that confer resistance to cefotaxime and sometimes ceftazidime and aztreonam were evolved from *bla*$_{OXA-10}$ (Evans and Amyes, 2014). Other variants of *bla*$_{OXA}$ enzymes have developed extended spectrum of activity to carbapenemes, and hence, called OXA-type carbapenemase. These carbapenemase like activity of *bla*$_{OXA}$ variants include *bla*$_{OXA-23}$, *bla*$_{OXA-24/40}$, *bla*$_{OXA-48}$, and *bla*$_{OXA-58}$. Most of the *bla*$_{OXA}$-ESBL variants were found in *A. baumannii* and *P. aeruginosa*, but *bla*$_{OXA-48}$ variants have only been isolated from clinically important strains of *Enterobacteriaceae* (Antunes *et al*., 2014; Evans and Amyes, 2014). Co-production of carbapenem-hydrolyzing metalloenzyme and an aztreonam-hydrolyzing *bla*$_{OXA}$ enzyme, can readily lead to resistance to all β-lactam antibiotics.

e. Minor groups of ESBLs

Other minor class A β-lactamases (PER, VEB, GES, BES, TLA, SFO, IBC) have also been discovered. Similarly, these groups are plasmid-mediated and/or integron associated (Bonnet, 2004; Dhillon and Clark, 2012; Naas *et al*., 2008). They are not simple point mutant derivatives of any of known β-lactamases and have been found in a wide range of geographic locations (Jones *et al*., 2009). Further information on these minor group ESBLs can be found elsewhere, the focus of this literature is only to ESBLs that are clinically and epidemiologically more important variants. Other novel β-lactamases, either chromosomally or plasmid encoded may continue to evolve further.

1.2.9 Epidemiology of ESBL-producing Bacteria

The prevalence of ESBL-producing Gram-negative bacteria is increasing worldwide (Logan and Weinstein, 2017) (Huang *et al*., 2006) (Bevan *et al*., 2017). The spectrum of activity and the diversity of ESBL encoding genes is also increasingly. Epidemiology of these organisms is now a main concern because of limited treatment options against them. The dissemination of resistant traits into a population of pathogenic *Enterobacteriaceae* and/or other non-fermentative Gram-negative bacilli is faster than the discovery of alternative antimicrobials.

Epidemiology of ESBL-producing *Enetrobacteriaceae* might need to be studied with a cutting-edge technology, whenever possible at each and different level of care (from a single patient, medical center, and to a wider geographic regions). Occurrence and dissemination of these strains strongly depends on some evolutionary phenomena or molecular mechanisms that may characterize the spread of ESBL-producing strains (Deurenberg *et al*., 2017).

Advancement in research skills and technology made the epidemiology of an infectious agent clearer than ever. The current use of next generation sequencing more frequently than before revealed that there are some unique epidemiological strains that have a potential to spread all over the world, frequently referred to as international clones (Guillard *et al*., 2014). Among *E. coli* strains frequently cited to be a cause of extra-intestinal infection (ExPEC) ST393, ST69, ST131, ST95, and ST73 were the common ones (Riley, 2014). These strains were identified in several regions of the world. Such strains with specific sequence types tend to feature similar dissemination and diseases (UTI, BSI and other extra-intestinal infections). Furthermore, these strains are associated with higher multidrug resistant property challenging treatment and care of patient.

Similarly, some strains of *K. pneumoniae* are unique in their global dissemination and cause of a disease. ST258, the most predominant KPC-harbouring clone, is one of the international clones that can cause serious infections. *K. pneumoniae* were most commonly described by their capsular types for virulence as classical and hypervirulent strain, capsular types K1 and K2 by conventional capsular typing and later KL1 and KL2 capsular locus (KL) based typing designates hypervirulent strains. Moreover, strains with corresponding genetic determinants for KL1 and KL2 were also related to other genetic determinants for hypercapsular phenotype. Though in-depth study is required at all levels of patient treatment and care such informative studies are confined to Europe, USA, and in some cases to South America. In LMICs, where the magnitude of the problem might be beyond extrapolation from a data generated elsewhere, it continues to be a challenge mainly because of resource limitation, and lack of highly trained and technical relevant personnel.

Mainly in Europe and USA, the available data shows that the prevalence of ESBL-producing strains is increasing and clonal dissemination is evidenced (Riley, 2014). Besides clonal

expansion of several distinct strains with defining epidemiological variables, shift of prevalent ESBL-genotypes from TEM, and/or *bla*$_{SHV}$ types to *bla*$_{CTX-M}$ types and further to specific *bla*$_{CTX-M}$- types has been oserved (Coque *et al*., 2008). Furthermore, carbapenemases have come into picture as a challenge in clinical care and public health.

In LMICs, mainly in Sub-Saharan African countries there is limited data. In Ethiopia, there are considerable number of phenotypic studies that show prevalence of antimicrobial resistance is high. These studies are confiened to the older medical University and Ehtiopian Public Health Institute (EPHI). However, these studies show very high prevalence of antimicrobial resistance the currently prescribed drugs in the country. Recently, a very high prevalence of antimicrobials has been reported from Ethiopia, 87% (Eshetie *et al*., 2015) from Bahirdar, 67% (Beyene *et al*., 2019) from EPHI, 78% (Gebre-egziabher, 2019) from University of Gondar, and 76% (Desta *et al*., 2016) from Addis Ababa University has been reported.
Comprehensive information regarding genomic epidemiology, clonality, molecular and strains level features are not yet sufficiently studied. Even when studied it might be underestimated or biased because of samller sample size or lack of accurate epidemiologic link. In one systematic review, the prevalence of ESBL was reported to be low (Tansarli *et al*., 2014). However, the review itself was limited to few studies, and hence, less likely to represent the actual burden of the problem in Africa.

1.2.10 Antimicrobial Resistance in *Enterobacteriaceae*

Enterobacteriaceae are an important family of bacteria s indicated earlier in this document are currently reconized well for the high prevalence of antimicrobial resiatnce to multiple, both first line and second line antimicrobials. They are unbiqutously found in environment, food, water and clinical environment. The antimicrobial resitance feature is astonishingly increasing in both developed and developing countries. WHO (2017) has recognized both the ESBL-producing *Enterobacteriaceae* and carbapenemase-producing *Enterobacteriaceae* as high priority and critical pathogens in human health. Also, the CDC(2019) report recognized ESBL-producing *Enterobacteriaceae* as serious threats to human health. Moreover, in the CDC, 2019 report despite suscces in reducing infections caused by antimicrobial resistant strains, the report noted that infections from ESBL-producing *Enterobacteriaceae* increased by 50%.

MDR strains of *Entrobacteriaceae* has become a major challenge in clinical environment, several of them reported from different nations with multiple antimicrobial resitance genes for Carbapenems (vanDuin and Doi, 2017), cephalosporin (Marr and Russo, 2019), aminoglycosides (Kim *et al*., 2018), fuoroquinolones (Roer *et al*., 2018) and colistin (Velasco *et al*., 2020).

Considering that *Enterobacteriaceae* are the prevalent causes of both community acquired and hopistal acquired infections, the increased rate of antimicrobial resitance among these group of bacteria has become a challenge for prescribers, public health specialists, and surgical proceudures, leaving vunarable segment of the population at greater risk.

1.2.11 Clinical Outcomes of ESBL-Producing Bacteria and Risk Factors

Various types of infections can be caused by ESBL-producing organisms. These infections includes urinary tract infection, bacteremia (primary or secondary) (Padmavathy *et al*., 2013), respiratory tract infection (nosocomial pneumonia, ventilator-associated pneumonia (Pilmis and Zahar, 2018), gastrointestinal tract infection (intra-abdominal abscess, peritonitis, cholangitis) (Reuland *et al*., 2012), skin and soft tissue infection, device related infection, sinusitis, neurosurgical meningitis related to ventricular drainage catheters (Oberoi *et al*., 2013).

Patients at high risk for developing colonization or infection with ESBL-producing organisms are often seriously ill patients with prolonged hospital stay and in whom invasive medical devices are present (urinary catheters, endotracheal tubes, central venous lines) for a prolonged duration (Feizabadi *et al*., 2010; Gudiol *et al*., 2010). Other risk factors including the presence of nasogastric tubes, gastrectomy or jejunostomy tubes; administration of total parenteral nutrition, recent surgery, hemodialysis, decubitus ulcers and poor nutritional status (Reuland *et al*., 2014).

Heavy antibiotic use is also a risk factor for acquisition of an ESBL-producing organism (Dzidic *et al*., 2008). Studies have found a relationship between third generation cephalosporin use and acquisition of an ESBL-producing strain (Baral *et al*., 2013; Kapil, 2005). However, the greatest risk factor for nosocomial acquisition of ESBL-producing strain is accommodation

in a ward or room with other patients with ESBL-producing organisms (Baral *et al*., 2013). Risk factors for colonization or infection with ESBL-producing organisms, especially the *bla*$_{\text{CTX-M}}$ producers include, history of recent hospitalization; treatment with cephalosporins, penicillins and quinolones; age greater than 65 years; and diabetes (Baral *et al*., 2013).

The risk of developing an infection from ESBL-producing and hypervirulent/invasive strains of *Enterobacteriaceae* is probably similar also for otherwise apparently healthy indiiduals, however, the severity of the disease is worse for patients with underlying chronic illness including Diabetes. Patients with diabetes have shown high-risk of developing pyogenic liver abscess from invasive/hypevirulent strains of *K. pneumoniae* (Marr and Russo, 2019).

Although there is no conclusive evidence, one potential source of colonization with the ESBL-producing strains in the community may be the use of veterinary oxyimino-cephalosporins like ceftiofur in livestock (Ewers *et al*., 2012; Kluytmans *et al*., 2012; Seiffert *et al*., 2013). The use of low-dose antimicrobials by the modern food animal industry as one of growth promoting substances and for prophylactic purpose than for cure is likely responsible for the emergence of multidrug resistant bacteria on the farms. These may reach the general population through human or animal carriers. Hence, misuse and overuse of broad-spectrum antibiotics, mainly cephalosporins must be a contributing factor for ESBL-producing *Enterobacteriaceae* in animals.

1.2.12 Microbiological Methods for Detection of ESBL

Detection of ESBL-producing Gram-negative bacilli begin with conventional microbiological tests. Cultivating the bacteria on an appropriate medium and isolating a pure colony of the bacteria from a clinical specimen is vital. Testing the bacteria for the resistance phenotype to extended spectrum cephalosporins can be done with two important steps. The first-step is screening, and testing for indicator cephalosporin of choice. This step allows cultivation of a pure isolate in the presence of an an indicator cephalosporin and checking for resistance or reduced susceptibility of bacteria to the indicator cephalosporin (Paterson and Yu, 1999).

The second-step, a confirmatory test begins with strains with reduced susceptibility to an indicator cephalosporin during screening and testing for relative susceptibility of the strain to

antimicrobial synergistic effect of a combination of oxyimino-cephalosporin and clavulanic acid as compared to the corresponding indicator cephalosporin alone. Reduced susceptibility to cephalosporin alone and increased zone of inhibition towards the inhibitor/clavulanic acid combination distinguishes the isolates as ESBL strain (Tenover *et al*., 1999; Paterson and Yu, 1999).

ESBLs detection methods, both the screening and confirmatory tests can be performed by disk diffusion, dilution methods, and modified automated systems. However, phenotypic detection of ESBL strains is difficult when the ESBL coexists with other potent β-lactamases like chromosomally encoded de-repressed AmpC, plasmid-mediated AmpC, and carbapenemases. Hence, confirmatory tests must be adjusted for multiple mechanisms of resistance to β-lactam antimicrobials in a single isolate (Poulou *et al.*, 2014). Methods widely performed at different laboratories for detection of only ESBLs phenotype are discussed below.

I. Phenotypic Tests

a. Disk diffusion method

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommends the disk-diffusion methods for screening ESBL-production among *Enterobacteriaceae*. It is possible to use disk diffusion methods for antibiotic susceptibility testing and screen for ESBL-production by noting specific diameters of zone of inhibition around the target antibiotic disks. According to the cut-off zone of inhibition set by standard guideline for AST, any reduced susceptibility to the screening antimicrobial will be considered for confirmatory test. Reduced susceptibility to one or several of the antimicrobials cefpodoxime, ceftazidime, aztreonam, cefotaxime or ceftriaxone by disk diffusion (indicator cephalosporins) should be considered as an initial point (Patil *et al*., 2011).

Since the affinity of ESBLs for different substrates vary, the use of more than one of these agents (indicators) for screening improves the sensitivity of detection. Cefotaxime is consistently susceptible to *bla*$_{\text{CTX-M}}$ (cefotaximase) and ceftazidime is consistently a good substrate for *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ variant enzymes (Winstanley and Courvalin, 2011). However, if a single drug must be used, cefpodoxime is the best single indicator. Susceptibility testing

only with cefpodoxime can lead to a high number of false positive results which might be due to mechanisms other than ESBL-production (Hope *et al*., 2007).

Though there is a difference in susceptibility of strains to these drugs depending on the enzyme produced by the bacteria, any strain with reduced susceptibility to any of these five agents indicates putative ESBL-production. Further confirmation of ESBL-phenotype can be performed by one of the phenotypic confirmatory tests.

b. **Dilution Methods**

Both EUCAST, and CLSI have proposed dilution methods for screening ESBL-production by *K. pneumoniae*, *K. oxytoca*, *E. coli* and *P. mirabilis*. Ceftazidime, aztreonam, cefotaxime or ceftriaxone can be used at a screening concentration of 1µg/mL or cefpodoxime at a concentration of 1µg/mL for *P. mirabilis* and 4µg/mL for other strains. Growth of bacteria at or above this antibiotic concentration can be considered as a phenotypic screening. Further confirmation of ESBL-production must be followed by growing the bacteria with similar concentration of antimicrobial both with and without inhibitor-β-lactamase molecules like clavulanic acid (EUCAST 2016 and CLSI 2016).

II. Confirmatory tests

a. Disk combination

The use of combination disks cefotaxime (30µg) or ceftazidime (30µg) disks with or without clavulanate (10µg) is recommended for phenotypic confirmation of the presence of ESBLs in *Klebsiella spp*., *E. coli*, *P. mirabilis* and *Salmonella spp*. (Polsfuss et al., 2012). According to the recommendation of CLSI for disk diffusion techniques test disks should be tested against confluent growth of bacteria on Mueller-Hinton agar. A difference of ≥5 mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/clavulanate disks is taken to be phenotypic confirmation of ESBL-production. For *Enterobacter spp*. *C. freundii*, *Morganella spp.*, *Providentia* and *Serratia spp*., it is better to use cefepime or cefpirome in the confirmatory tests as they are less prone to attack by the chromosomal AmpC-β-lactamases that may be induced by clavulanate in these species (Winstanley and Courvalin, 2011).

b. Mast® D68C test

It is important to detect ESBL-phenotypes in strains of *Enterobacteriaceae* that co-express the AmpC resistance. In this test a combination of cefpodoxime with and without ESBL-inhibitors and AmpC-inhibitor is sorted into four different cartridges. Cartridge A: contains 10µg disc cefpodoxime, cartridge B: contains10µg disc cefpodoxime plus ESBL-inhibitor disc, cartridge C: contains 10µg disc cefpodoxime plus AmpC-inhibitors, and cartridge D: contains 10µg disc cefpodoxime plus ESBL-inhibitors plus AmpC-inhibitors (Nourrisson *et al*., 2015). The interpretation of the test is based on the difference of the zone of inhibition between the different discs used.

c. Broth microdilution

Confirmatory testing can also be performed by broth-microdilution using ceftazidime(0.25-128µg/mL), ceftazidime plus clavulanic acid (0.25/4 - 128/4µg/mL), cefotaxime (0.25-64µg/mL), or cefotaxime plus clavulanic acid (0.25/4 - 64/4µg/mL)(Queenan *et al*., 2004). Broth microdilution is performed using standard methods. Phenotypic confirmation is considered as ≥3 twofold serial dilution decreases in minimum inhibitory concentration of either cephalosporin in the presence of clavulanic acid compared to its MIC when tested alone (Cormican *et al*., 1996).

Cefoxitin susceptibility in isolates with positive screening tests but negative confirmatory tests was suggested as a means of deducing the mechanism of resistance (Steward *et al*., 2001). ESBL-producing isolates appear susceptible, while those with plasmid AmpC enzymes are resistant. However, resistance to cefoxitin seems to be increasing in ESBL-producing isolates due to efflux or permeability changes or coexistence of ESBLs with AmpC enzymes.

Based on interpretive guidelines of standardizing organizations like EUCAST and CLSI, isolates which have a positive phenotypic confirmatory test should be reported. The MIC of other related cephalosporins should be checked and if appropriate, it might be used. Penicillins (piperacillin or ticarcillin) are reported as resistant regardless of MIC, but β-lactam/β-lactamase inhibitor combinations (for example, ticarcillin-clavulanate and piperacillin-tazobactam) are reported as susceptible if MICs/zone diameters are within the range.

III. Modified/Automated ESBL detection

Several tests have been developed and being developed to improve the detection of ESBL-producing strains. Some of these tests are automated and advanced or improved the turnaround time of tests. These tests significantly simplified the bulk of conventional methods. However, these new/modified methods incur additional costs and are technically demanding for resource limited countries. Moreover, the basic principles remain the same and no improvements were reported regarding differential detection ESBL-phenotype in multiple β-lactamase producing isolates as compared to the classical/ conventional methods.

a. Chromogenic Media

This method relies on the selective ability of the media and chromogenic substrate that builds up to give color as the bacteria grows to produce an enzyme that target hydrolysis of the chromogen. It is a rapid method that combines identification of the bacteria and presumptive detection of the ESBL-phenotype. Addition of selective antibiotics to a chromogenic media is a breakthrough for some strains like MRSA. But, it is very challenging for *Enterobacteriaceae*. It requires color based differentiation of several genera of Gram-negative bacilli harboring ESBL, maximal suppression of growth of highly complex and abundant resident flora for fecal samples, and an indicator of β-lactams that offers maximal coverage of ESBL-spectrum (Gazin *et al*., 2012).

Commercially available chromogenic media for detection of ESBL producers include chromID ESBL (bioMérieux), Brilliance ESBL (Oxoid Ltd., Basingstoke, United Kingdom), and CHROMagar ESBL (CHROMagar, Paris, France) (Blane *et al*., 2016). The chromID and Brilliance ESBL are ready to use media, but CHROMagar ESBL needs to be prepared by adding selective mix to the CHROMagar agar base. CHROMagar CTX supplement should be added for detection of $bla_{\text{CTX-M}}$ producers. Performance and characteristics of the media should be assessed both by for analytical and clinical studies (Blane *et al*., 2016; Gazin *et al*., 2012).

b. VITEK-ESBL

The VITEK-ESBL test (bioMerieuxVitek, Hazelton, Missouri) utilizes both cefotaxime and ceftazidime. The test setup is the drug alone (at 0.5µg/mL) and in combination with clavulanic acid (at 4µg/mL). Inoculation of the cards is identical to that performed for regular VITEK

cards (Dashti *et al*., 2006). Analysis of all wells is performed automatically once the growth control well has reached a set threshold (4-15 hours of incubation). A predetermined reduction in the growth of the cefotaxime or ceftazidime wells containing clavulanic acid, compared with the level of growth in the well with the cephalosporin agent alone, indicates presence of ESBL. Some evidences show that sensitivity and specificity of this method may exceed 90% (Chen *et al*., 2009; Spanu *et al*., 2006).

c. Etest

The Etest ESBL strip (bioMerieuxVitek, Hazelton, Missouri) carries two gradients: on the one end, ceftazidime; and on the opposite end, ceftazidime plus clavulanic acid (Dashti *et al*., 2006). MIC is interpreted by reading at the point of intersection of the inhibition eclipse with the Etest strip edge. A ratio of ceftazidime MIC to ceftazidime-clavulanic acid MIC equal to or greater than 8 indicates the presence of ESBL (Aggarwal and Chaudhary, 2004). The reported sensitivity of the method as a phenotypic confirmatory test for ESBLs is 87% to 100%, and the specificity is 95% to 100% (Cormican *et al*., 1996)

d. MicroScan panels

MicroScan panels (Dade Behring MicroScan, Sacramento, CA) comprise dehydrated panels for micro-dilution antibiotic susceptibility testing. Those used for ESBL detection contain combinations of ceftazidime or cefotaxime plus β-lactamase inhibitors. The method has received approval from Food and Drug Administration. In studies conducted on large sample size ESBL-producing isolates, it appeared that the method is reliable (148,149).

e. Becton Dickinson (BD)

Becton Dickinson Biosciences (Sparks, Md) have introduced a short incubation system for bacterial identification and susceptibility testing, known as BD Phoenix(Lupetti *et al*., 2009). The Phoenix ESBL test uses growth response to cefpodoxime, ceftazidime, ceftriaxone, and cefotaxime, with or without clavulanic acid, to detect the production of ESBLs. Results are usually available within 6 hours (Lupetti *et al*., 2009). The BD Phoenix ESBL detection method detected ESBL-production in greater than 90% of strains genotypically confirmed to produce ESBLs. The method correctly detected ESBL-production by *Enterobacter spp.*,

Proteus spp. and *Citrobacter spp*., in addition to *Klebsiella* spp. and *E. coli* (Chung *et al*., 2009; Lupetti *et al*., 2009).

Generally, there are several phenotypic tests known today. Many commercial tests built technically simplified on the basis of colorimetry and lateral flow principles were innovated. Some of these tests include: ESBL-NDP (Nordmann *et al*., 2012), Nitrocefin test (Vergis *et al*, 2018), and Carba5 (Boutal *et al*., 2018). All these tests have their own advantages and disadvantages. Several generations of such tests can be innovated and may be produced. Nevertheless, there is no single best confirmatory test that suffices all conditions.

IV. Molecular methods

Molecular characterization of a genetic mechanism underlying a given phenotype is an integral part of clinical, epidemiological and any form of information needed for public health. It might demand comprehensive and detail approach where epidemiological surveillance or an outbreak detection is needed. Phenotypic studies lack the necessary depth required to support optimal treatment options and control strategies for possible outbreaks involving resistant strains. Molecular detection and identification clinically important bacterial strains might be essential for a reliable epidemiological investigation of antimicrobial resistance (Empel *et al*., 2008; Jones *et al*., 2009).

Classical molecular detection is usually based on amplification of target gene by PCR. Then followed by sequencing for analyzing variants or mutants of the amplified gene. Thus, it largely relies on the use of specific primers, allowing the amplification of a given family or group of genes and their members. Further improvements in the method of detection and quantitation of the target gene, and a type of genome to be amplified (DNA/RNA) have led to the generation of several of improved molecular techniques that can be used for detection of specific genes and identification of pathogens. Over the years; development, and usage of molecular methods in medicine and other areas of human endeavor have shown great advancement. The widely used methods include: the classical PCR (RT-PCR, LAMP, and RPA), RFLP, PFGE, DNA-microarray, and WGS. Molecular methods are now important tools for studying cause of an outbreak, clonal dissemination of resistant strains, virulence and resistanc genes. Principles and applications of commonly used molecular methods are described below.

a. Polymerase chain reaction (PCR)

It is a straightforward approach to genetic detection of β-lactamases or direct amplification of the whole β-lactamase (*bla*) gene or its parts using PCR with oligonucleotide primers that are specific for the target gene. The gene sequences of single enzyme families are available in public databases such as GenBank (National Center for Biotechnology Information, http://ncbi.nlm.nih.gov/Genbank).

PCR enables determination of individual classes of β-lactamases but can neither discriminate between enzymes with narrow and extended spectrum activity (*bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{OXA}$ types) nor distinguish between variants/enzymes. Therefore, other genetic based identification of specific point mutations that extend the substrate specificity were introduced in to ESBL detection (Foley *et al*., 2009). There are modified PCR approaches with both detection and quantitation. RT-PCR with specific oligonucleotide probe detections may also give some details in to the sequence of the amplicon.

b. PCR-RFLP

The RFLP analysis enables characterization of DNA after digestion using restriction endonucleases and subsequent separation of DNA fragments in agarose gel. Restriction endonucleases are enzymes that cut double stranded or single stranded DNA at specific recognition of nucleotide sequences known as restriction sites. Discrimination of various DNAs is based on restriction fragment length polymorphism. This polymorphism occurs due to the presence or absence of restriction sites. The RFLP method is also simple and rapid for the detection of known mutations that alter recognition sites of restriction endonucleases (Foley *et al*., 2009).

c. PFGE genomic and plasmid DNA

This technique overcomes the limitations of restriction endonuclease assays. It is a variation of agarose gel electrophoresis in which the orientation of the electric field across the gel is changed periodically. This modification enables large fragments to be effectively separated by size. A restriction endonuclease enzymatically cuts DNA at a specific nucleotide recognition sequence. The number and sizes of restriction fragments are influenced by the recognition

sequence of enzyme and composition of DNA. Bacterial DNA is digested with endonucleases that have relatively frequent restriction sites, thereby generating hundreds of fragments ranging from ~0.5 to 50 kb in length. Such fragments can be separated by size using agarose gel electrophoresis. The pattern stained by ethidium bromide and examined under UV light. Different strains of the same species have different restriction endonuclease profiles because of variations in their DNA sequences (Goering, 2010; Ribot *et al*., 2006).

Nevertheless, both PCR-RFLP and PFGE (genomic and plasmid) are not good candidates for ESBL-detection at this age. Because this tests are expensive, complex, and time consuming. There are multitude of simplified alternative methods to serve this purpose, and here these methods are mentioned for theoretical possibility.

d. DNA microarray

The major disadvantage of the above methods is the limited number of targets that can be detected and differentiated in each reaction. DNA microarray is a promising genotyping technique with a high multiplexing ability. Instead of detecting and studying one gene at a time, microarrays allow thousands or tens of thousands of specific DNA sequences to be detected simultaneously. DNA microarrays are used in three major clinical areas: (a) for gene expression profiling, measuring the expression level of thousands of genes in any tissue sample, (b) for genotyping determination of disease relevant genes or agents causing diseases, and (c) DNA sequencing screening thousands of DNA base pairs for mutations in specific genes for which the normal sequence is already known (for screening single nucleotide polymorphisms, SNPs).

The DNA microarray technology is based on glass slides that were spotted with thousands of specific DNA probes based on genes present on reference genome. As whole-genome data for a species is available the microarray probes will increase to include the accessory genes probes that were not previously available in the pan-genome of the reference strain. Because it uses glass slide and florescent dyes the technique is highly sensitive and specificic, and for the same reason it is time consuming and expensive (Anjum *et al*., 2017).

Because β-lactamases are characterized by a wide genetic diversity, an exhaustive enzyme characterization; using a PCR approach can be time consuming, and a microarray approach

has greater ability in detecting simultaneously several ESBL encoding genes from a single strain (Ballarini *et al*., 2012).

e. Whole genome sequencing (WGS)

An advantage of WGS over other molecular methods is that it has a potential to cover many different targets at the same time and to subtype specific gene variants. WGS is very important in this era where antimicrobial resistance is a global agenda and therapeutic options are limited. Tools like next generation sequencing that provide robust data on genome of a pathogen are important to salvage some pathways that can be targeted for therapeutic options especially in MDR/XDR/PDR strains (Hawken and Snitkin, 2019).

Currently there is huge advancement in sequencing technology. An improvement and advance in technology have put sequencing at prime position of public and medical research. Microbiological studies including antibiotic resistance, tracking dissemination/transmission clones, and tracking pathogens at animal-human interface has become possible. Detection of resistance, surveillance, typing, and subtyping is easier than before. Though the cost of sequencing is still high, it is promising in that it is decreasing over years. Modern genome sequencing relies on next generation sequencing (NGS) platforms and/or a combination of second or third-generation sequencing technologies. Although the entire genome is sequenced, it cannot be assembled completely. A genome contains repeated stretches of DNA that are longer than the length of DNA that can be sequenced in a single read by the sequencing technology employed.

Evolution of sequencing technologies: Genome sequencing has developed very fast over the last three decades. Though there were several attempts to sequence a DNA or RNA, the real birth of sequencing was first introduced by Sanger *et al*, in 1977. Because they used the dideoxy nucleotide in addition to the deoxynucleotide the method is often called dideoxy- or chain termination method (Wong, 2013).

Since then, sequencing techniques and technologies were under continuous improvements with advancement in technology. Further advancements in techniques and knowledge about genomics lead to the development of latest sequencing technologies. Moreover, the demand to answer biological questions has fueled the evolution and revolution of the sequencing methods

(Koboldt *et al*., 2013). For simplicity of understanding of the sequence of evolution, principles, technological advancements and applications of the sequencing technologies; they are often grouped as first-generation, second-generation, and third-generation sequencing (Heather and Chain, 2016).

First-generation sequencing: Sanger and Maxam-Gilbert technologies are called first-generations sequencing technologies. Sanger sequencing is performed by using chemically modified nucleotides, dideoxy-nucleotides (ddNTPs). Each of these ddNTPs correspondingly labeled for ach DNA bases as dideaoxy-A, -T, -G, -C. The dideoxy-nucleotides are used with dNTPs for elongation of nucleotide chains. Once the ddNTPs are incorporated into the DNA strand they prevent elongation of the sequence and the nucleotides chain terminates. When the chains terminate several DNA fragments of different size will be obtained, then these fragments will be separated according to their size variation using gel slab where the resultant bands corresponding to DNA fragments can be visualized by imaging system (Heather and Chain, 2016; Kchouk *et al*., 2017).

Maxam-Gilbert sequencing also called chemical degradation sequencing is based on cleaving of polynucleotides by using chemicals. It was effective method of sequencing short nucleotide polymers. Chemical degradation generates breaks at short chains of one or two of the four nucleotide bases in each round of the four reactions (C, T+C, G, A+G. A chemical degradation generates a series of marked fragments that can be separated according to their size variation by electrophoresis. However, the method is unsafe, it uses highly toxic and radioactive chemicals (Kchouk *et al*., 2017).

Second-generation sequencing (SGS): Second-generation sequencing sometimes referred to as massive parallel sequencing, these are a group of the first wave of next-generation sequencing (NGS) that provide sequencing of whole genome of pathogens in one sequencing run. The sequencing may be either a genome of bacterial isolates of different patients, or multiple species present in a sample from one individual (metagenomics). Several second-generation sequencing platforms available each with its own advantage and disadvantage include: ABI sequencing, Roche/454 sequencing, Ion torrent sequencing, and Illumina (Solexa) sequencing (Heather and Chain, 2016).

An advantage of SGS as compared to Sanger sequencing is that a single protocol can be used for all pathogens for both identification and typing applications. This comparative advantage make SGS an ideal tool for clinical microbiology and infection prevention strategies (Deurenberg *et al*., 2017). Unlike Sanger sequencing target specific primers are not required for SGS. Before sequencing, the genome of the pathogen needs to be fragmented because the whole genome cannot be sequenced at a full length stretch as one part. An average fragment length of a DNA that a second-generation sequencers can sequence varies from 100-300 bases (Levy and Myers, 2016). Second-generation sequencing requires preparation of libraries in which fragments of DNA or RNA are fused to adapters and barcodes to distinguish the DNA of the sequenced isolates after sequencing, followed by a clonal amplification, normalization, and sequencing. Hence, robust preparation of libraries which contains a representative source of the DNA/RNA of the genome under investigation is needed (Kchouk *et al*., 2017; Yohe and Thyagarajan, 2017).

Fragmentation of the genome can be performed in several ways. Either mechanical, Adaptive Focused Acoustics (AFA) technology or enzymatic fragmentation by using transposons as in Nextera-XT library preparation kit from Illumina and then followed by adaptor ligation. Enzymatic method has an advantage in that fragmentation and fusion of the adaptors to a DNA/RNA fragments can be performed in one step (Deurenberg *et al*., 2017).

The benefits of SGS methods include use of small concentration of DNA, mechanical fragmentation has advantage for generation of the appropriate fragment length that is less influenced by factors in the sample and might inhibit enzymes used during library preparation. It is therefore suitable for preparation of a library directly from a sample material such as biopsies and feces (Deurenberg *et al*., 2017).

Second-generation sequencing (SGS) technologies have offered many improvements over Sanger sequencing. Nevertheless, limitations in short read lengths make them poorly suited for some particular biological problems including need for rigorous assembly and determination of complex genomic regions, gene isoforms detection, and methylation detection (Buermans and Dunnen, 2014).

Third-generation sequencing: microbial genomes are complex with many repetitive regions that short read second generation sequencing cannot overcome challenges in genome assembly. The shortcomings in SGS lead scientists to further develop sequencing technologies to overcome these challenges. Because PCR amplification is not needed and thus sequencing time significantly reduced, third-generation sequencing technologies provide low sequencing cost and easy sample preparation (Levy and Myers, 2016). Moreover, third-generation sequencing are able to produce long reads, and provides ideal option for sequencing several kilobases long parts of the genome and improves resolution of the assembly problem and repetitive regions of complex genomes as compared to second-generation sequencing (Rhoads and Au, 2015). Third-generation sequencings such as the MinION (OxfordNanopore) and the PacBio (Pacific Biosciences) can generate larger fragments up to 200 kb (Kchouk *et al*., 2017; Levy and Myers, 2016). These sequencers are not yet fully used in clinical microbiology laboratory due to unaffordable cost, lower quality of sequences at individual base level and the low throughput. To overcome this, many hybrid sequencing strategies have been developed to make use of more accurate short reads in conjunction with PacBio long reads. Hybrid sequencing strategies are more affordable and advantageous than using PacBio sequencing alone (Kchouk *et al*., 2017).

Pathogen typing methods based on core genome sequences were found to be reliable segment of a genome than including accessory genes. Yet, bioinformatics, genome assembly, and some other downstream analysis of the sequence data are a challenge. Furthermore, parts of the genome may still be excluded from analysis by draft sequence trimming based on sequence quality parameters for reliable analysis and interpretation (Levy and Myers, 2016).

1.2.13 Treatment of ESBL-producing Bacteria

Extended-spectrum cephalosporins are drugs of choice for treatment of Gram-negative pathogens. Because of their safety and efficacy, these agents have been extensively used since their discovery. Such extensive use of the extended-spectrum cephalosporins commonly called third-generation cephalosporins might have served the generation of several different types of bacterial species able to produce ESBL enzymes. These enzymes in turn can inactivate these group of antimicrobial agents and hence, leading to the evolution of resistant strains – non-

susceptible to previous dose of antimicrobial treatment. Thus, infections caused by ESBL-producing strains are major challenges in the world (Bush and Bradford, 2016; Riley, 2014).

ESBLs exhibit hydrolytic activity against penicillins, cephalosporins (first, second and third generation) and monobactams, but limited hydrolysis to carbapenemes, cefepime and cephamycins. Class-A β-lactamases are highly susceptible to inhibition by β-lactamase inhibitors (BLIs) including clavulanate, sulbactam, and tazobactam (Bush and Bradford, 2016). There are few options to treat patients infected with ESBL strains. The primary options are carbapenems, and non-carbapenem β-lactams alone and /or combination of β-lactam-β-lactam inhibitors can also be used under some specific clinical conditions (Paterson, 2007).

a. Carbapenems

Carbapenems are first-line and most reliable antimicrobial agents of choice for treatment of severe/serious infections from ESBL-producing strains. Then, extensive use of these agents over the last few decades might have contributed to the emergence of carbapenem resistant strains of *Enterobacteriaceae* (CRE) and further fueling the development of more resistant strains (Tängdén and Giske, 2015). These CRE strains were very often resistant to all β-lactams by acquiring genetic determinants for resistance mechanisms to other antimicrobial classes too. The production of carbapenemase enzymes encoded by mobile genetic elements that inactivate carbapenems and other β-lactam agents are predominant mechanisms that confer resistance (Lutgring and Limbago, 2016).

Enterobacteriaceae can also produce enzymes such as AmpCs and ESBLs that do not readily inactivate carbapenems on their own, but can confer carbapenem resistance when combined with chromosomal porin mutations. Considering the increase in carbapenemase mediated resistance and that carbapenem is the last resort drug for serious/complicated infections/disease from ESBLs; there must be carbapenems sparing alternative treatment options at least for mild infections (Pranita and Rodriguez-ba, 2017).

b. Non-carbapenem β-lactams

As primary alternative of carbapenem sparing, non-carbapenem-β-lactams are the major targets. However, a recent review by Bano and his colleagues reported that that the use of

cephamycins (cefepime), piperacillin-tazobactam, ceftolozane-tazobactam, and ceftazidime-avibactam shows conflicting results.

ESBLs are usually MDR strains and may bear other resistance mechanisms (Pranita and Rodriguez-ba, 2017). When there is mutation with outer membrane proteins and plasmid-mediated AmpC cephalosporinases, these agents may not be appropriate candidates to replace carbapenem. Though this mechanisms also affect susceptibility of these strains to carbapenems as well.

Cefepime is an oxyimino cephalosporins with increased resistance to degradation by β-lactamases. The effectiveness of cefepime diminished in infections with higher bacterial inoculums and poor outcome also related to failure to meet pharmacodynamic targets due to inadequate dosing/scheduling. Similarly, studies on the use of piperacillin-tazobactam for ESBL-producing Gram-negative bacilli show contradicting results (Lee *et al*., 2007; Pranita and Rodriguez-ba, 2017). In one study, piperacillin-tazobactam showed higher activity with non-ESBL *K. pneumoniae* strains at higher load of bacteria. Nevertheless, it cannot maintain the same activity with ESBL-producing *K. pneumoniae,* and second factor is a problem with dosing and scheduling. For these reasons using piperacillin-tazobactam for treatment of infections caused by ESBL-producing strains is debated, and yet no conclusive evidence is available (Sader *et al*., 2007).

c. β-lactam/β-lactam inhibitors

The β-lactam inhibitors bind to β-lactamases to inactivate these enzymes. β-lactam inhibitors commonly used include clavulanic acid, sulbactam, and tazobactam. Newer generations of β-lactam inhibitors that bear improved spectrum of activity over the classical β-lactam inhibitors were discovered and approved for clinical use as cefepime-tazobactam, and ceftriaxone-sulbactam.

Ceftazidime-avibactam, ceftolazone-tazobactam and meropenem-vaborbactam were recently approved for complicated intra-abdominal infections together with metronidazole, and for complicated UTI (Docquier and Mangani, 2018; Sharma *et al*., 2016). In one case study, meropenem-vaborbactam showed better efficacy against ceftazidime-avibactam resistant *K.*

pneumoniae that caused bacteremia and abscess in a liver transplant recipient (Athans *et al*., 2019).

β-lactam inhibitors have little antimicrobial activity when used alone but when combined with other β-lactam antibiotics, they increase the spectrum of activity and stability of the antibiotic against β-lactamases.

Combination of β-lactam and β-lactam inhibitors can be possible alternative antimicrobial agents for sparing carbapenem. However, decreased efficacy because of inoculum effect or at higher bacterial load, presence of multiple other β-lactamase enzymes (genes/co-resistance gene), and inadequate conventional dosing were some of the obstacles reported in studies (Lee *et al*., 2007; Sader *et al*., 2007; Pranita and Rodriguez-ba, 2017).

Inoculum effect: piperacillin-tazobactam was compared for efficacy/spectrum of activity against ESBL and non-ESBL strains with same MIC. Piperacillin-tazobactam showed higher efficacy for non-ESBL strains, where it failed to do the same for ESBL-strains. Whereas, amoxicillin-clavulanate has overcome the inoculum effect and performed better against ESBLs (Sader *et al*., 2007). The significance of the inoculum effect in clinical infections is not known.

Multiple resistance: ESBL genes are acquired sometimes from plasmids that contain other β-lactamases. Complete information about other resistance genes including narrow spectrum β-lactamase genes in which the ESBL-mediated resistance occurs influences the choice of βL/βLI combination and treatment outcome (D'Angelo *et al*., 2016). Extended spectrum β-lactamase producing strains may acquire plasmid mediated AmpC genes and can be resistant to clavulanate or tazobactam. Non-ESBL parental β-lactamase enzymes like $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-1}}$ may also coexist with ESBL enzymes, and might be over expressed and overcome the activity of βLIs (Bedenic *et al*., 2015; Harris *et al*., 2015; Karen and Jacoby, 2010). Variation in ESBL types can also affect their susceptibility to βL/βLI, $bla_{\text{CTX-M}}$ variants which are inhibited by tazobactam than sulbactam and clavulanate. The susceptibility further decreased with the presence of bla_{CMY}-like and bla_{SHV} type enzymes. Furthermore, the resistance might arise from efflux mechanisms and decreased membrane permeability (Harris *et al*., 2015).

Conventional dosing: conventional dosing with βL/βLI might not always achieve optimum pharmacokinetic and pharmacodynamic indices (Harris *et al*., 2015). Recently reviewed papers

on treatment of ESBL strains are converging on the concept of measure of efficacy of combination of βL/βLI as the peak concentration is less critical than the duration of exposure to the drug at a concentration above the MIC (Docquier and Mangani, 2018; Harris *et al*., 2015; Pranita and Rodriguez-ba, 2017). Thus, duration of exposure/scheduling the therapy is critical for use of βL/βLI combinations for treatment of infections caused by ESBL strains.

d. Non-β-Lactam Antimicrobials

Aminoglycosides (amikacin, gentamicin, tobramycin), and floroquinolone (ciprofloxacin and levofloxacin) can be used for the treatment of ESBL-producing strains when active in vitro. However the ESBL strains are MDR strains that may harbor resistance determinant factors for both other-β-lactams and non-β-lactam antimicrobials (Son *et al*., 2018).

In addition, the plasmids bearing genes encoding ESBLs frequently carry genes encoding resistance to other antimicrobial agents, such as trimethoprim, sulfonamides, tetracycline-derivatives (glycylcyclines, tigecycline, and eravacycline) and chloramphenicol (Giske *et al*., 2008; Lee *et al*., 2006).

There were several reports of plasmid-encoded decrease in susceptibility to fluoroquinolones often associated with plasmid-mediated cephalosporin resistance (Dhillon and Clark, 2012; Guillard et al., 2014; Isturiz, 2008; Liu et al., 2013). There appears to be strong association between fluoroquinolone resistance and ESBL-production even in the absence of plasmid-encoded decrease in fluoroquinolone susceptibility, although the reason for this association is not well understood (Rupp and Fey, 2003). Fluoroquinolone may be used for treatment of uncomplicated urinary tract infections when found susceptible but increased probability of co-resistance of ESBLs with quinolones limits the role of these antibiotics (Liu *et al*., 2013). Carbapenems were found to be superior to fluoroquinolones for treatment of serious infections caused by ESBL-producing organisms (Isturiz, 2008).

1.2.14 Prevention and Control of ESBL-producing Bacteria

Proper infection control practices and barriers are essential to prevent spreading and outbreaks of ESBL-producing bacteria (Kizilca *et al*., 2012). The reservoir for these bacteria seems to be the gastrointestinal tract of patients. Alternative reservoirs could be the oropharynx, colonized

wounds and urine (Adler et al., 2012). The contaminated hands and stethoscopes of healthcare providers are important factors in spreading infection between patients (Cantey *et al*., 2013).

Essential infection control practices should include avoiding unnecessary use of invasive devices such as indwelling urinary catheters or IV lines, hand washing by hospital personnel, increased barrier precautions, and isolation of patients colonized or infected with ESBL producers (Cantey *et al*., 2013; Nuotio *et al*., 2012). At institutional level practices that can minimize the spread of such organisms include clinical and bacteriological surveillance of patients admitted to intensive care units and antibiotic cycling as well as policies of restriction especially on the empirical use of broad spectrum antimicrobial agents such as the third- and fourth-generation cephalosporins and fluoroquinolones (Bradford, 2001).

In general, public health risks caused by ESBL-producing bacteria are determined by the frequency of the occurrence (prevalence) in human and animal population, the genetic characteristics of the β-lactamase genes involved, and the transmission from animals or animal products to humans. Mitigation measures should therefore aim to reduce the prevalence in humans, animals, and to reduce transmission from contaminated animals/animal products to humans.

There were no data on the comparative efficiency of individual control measures in reducing public health-risks from ESBL-producing bacteria in relation to food producing animals. Prioritization of control measures might be complex in the absence of enough evidence and the effectiveness of measures should also be based on the best available evidence. However, in countries like Ethiopia comprehensive data that can dictate appropriate control measures are hardly available.

1.3 Statement of the Problem

The origin of resistance might be a natural process whereby the resistance genes are maintained in nature by the presence of antibiotic producing bacteria in soil or acquired from other bacteria through horizontal gene transfer. Resistance to penicillin initially was due to production of the enzyme penicillinase by bacteria, following that second-generation of penicillins, like cloxacillin were developed to overcome such resistance.

Use of these antibiotics again forced the bacteria in turn to alter the target site for binding of β-lactam antibiotics (from PBP2 to PBP2a, an enzyme with low affinity for β-lactam amide bond) and this led to the development of another resistant phenotype called methicillin resistant *S. aureus* (MRSA) (Tenover *et al*., 1999). Several generations, including newer generations of cephalosporins were developed with a wide range of activity and resistance to existing known β-lactamase enzymes. Further developments introduced 3rd and 4th generation cephalosporins. These newer generations of cephalosporins were resistant to the then identified β-lactamases produced by the Gram-negative-bacteria (Tenover *et al*., 1999; Jacoby and Munoz-Price, 2005). However, some species of Gram-negative bacilli were identified again with resistance phenotype to these new antimicrobials. The mechanism of resistance was studied and elucidated, these bacilli produced extended-spectrum β-lactamases (ESBLs). The genetic determinants of the resistance/enzymes are usually carried on mobile genetic elements like plasmids. The dissemination of these gene and subsequently the resistance property was very rapid, and their prevalence is increasing globally.

The burden of ESBLs varies based on several factors ranging from patient factors, infection prevention policies, rational drug use policies, to national and international factors. Challenges imposed on clinical care and/or public health from infections/diseases associated with ESBL strains were studied and are being studied mostly in high-ncome countries. The findings of these studies revealed that the problem is increasing and posing a challenge for clinical practice (Adler *et al*., 2012; Aggarwal and Chaudhary, 2004).

A global surveillance from Europe, North and South America, and Asia, estimated the detection frequencies for ESBL-producing *K. pneumoniae* and *E. coli* isolates to be between 2.2-44% (Adler et al., 2012). Other studies from high-income countries have also shown that the rate of isolation of ESBL-positive bacteria was higher in clinical settings. The burden of infection from these bacteria varies from one geographical region to the other (Ahmed-Bentley *et al*., 2013; Dai *et al*., 2013; Dhillon and Clark, 2012). There are limited studies in Low and Middle Income Countries (LMICs), few studies from Africa, particularly from South Africa (several but no national surveillance), and Guinea Bissau (32.6%) reported high prevalence and also noted that strains are clonally diversified (Isendahl *et al*., 2012). Recently, a study conducted in Mali reported a higher rate of ESBL in both *E. coli* (64.5%) and *K. pneumoniae*

(76.9%) among bacteremic patients. Few previous phenotypic studies from our setting reported a prevalence of 38% among *E. coli* and *K. pneumoniae* (Shewki Moga Siraj *et al*., 2014), and 46% of *E. coli* (Mulualem *et al*., 2012) strains were ESBL-producing.

Antimicrobial resistance containment requires improvements in infection control strategies, infection prevention policies, adherence to drug policies, epidemiological evidence, and other relevant information for local consumption. Furthermore, regional, and national surveillance of resistant strains may generate pertinent evidence that may help for planning from local through regional to global collaborative intervention strategies. Nevertheless, in regions where access to health care is less, information is limited, capacity of the health care delivery is limited, and quality of antimicrobial agents used are not guaranteed and where microbiological diagnostic facilities are scarce, the burden of antimicrobial resistance might be beyond extrapolation from other data sources like global reports or elsewhere.

Availability of epidemiologic data and strict surveillance of high-risk clones may enhance the capacity for disease prevention and rational use of antimicrobial agents. More researches are needed to involve to develop low cost, high-impact intervention strategies for control of antimicrobial resistance in developing countries (Okeke *et al*., 2007). The choice of empiric antibiotics should also take into account local epidemiology of strains, resistance patterns and availability of antimicrobial options within that setting (Furyk *et al*., 2011).

Hence, we aimed to study genomic epidemiology and molecular features of ESBL-producing *Enterobacteriaceae*. The case of Jimma Medical Center, a tertiary level teaching hospital in Ethiopia. Thus, the data might be a baseline for futire studies and may alert Ministry of Health, and all other stakeholders who work on antmicrobial resitance. .

1.4 Significance of the study

In Ethiopia, there are few studies on the burden and dissemination of resistance determinant genes, virulence, and disease causation by ESBL-producing bacteria. Establishing local baseline data with currently available technology regarding ESBL-producing strains is important to develop, reconsider, or improve treatment guidelines, infection control policies and epidemiologic strategies. Global outbreaks have been reported and pandemic lineages

reported, therefore detecting such lineages and containment of such outbreaks is mandatory. Therefore, the present study was undertaken to determine the prevalence and molecular characteristics of ESBL-producing bacteria among patients at JMC, a tertiary care hospital where we do not have sufficient data regarding the complete picture of epedimiology of antimicrobial resistance. Resistant strains were identified, and spectrum of antimicrobial resistance was elucidated. More information with necessary depth is generated for the most common *Enterobacteriaceae* and most prevalent strains, *E. coli* and *K. pneumoniae.* This study is important for JMC as an epidemiologic dataset regarding the circulating strains burden of ESBL-producing bacilli in the hospital. It can be used as baseline for more comprehensive future research, and most importantly for planning of an antimicrobial stewardship program at the study site.

1.5 Hypothesis

1. The prevalence of extended spectrum β-lactamase-producing Gram-negative bacilli strains at JMC/Ethiopia is different form the prevalence of extended-spectrum β-lactamase-producing strains from Europe, USA and elsewhere.
2. The predominant extended spectrum β-lactamase genes from strains isolated at JMC is different from ESBL-genes from strains isolated elsewhere.
3. There is clonal expansion of specific strains of extended spectrum β-lactamase-producing *Enterobacteriaceae* at JMC patient population.

1.6 Objectives of the study

General objective

- To determine genomic epidemiology and molecular characteristics of extended-spectrum β-lactamase-producing Gram-negative bacilli (*Enterobacteriaceae)* at Jimma Medical Center (JMC), Jimma, Ethiopia.

Specific objectives

- To determine prevalence of ESBL-producing *Enterobacteriaceae* and non-fermenters isolated from different clinical specimens at JMC.

- To determine the predominant genotypes of extended spectrum β-lactamase among strains of *Enterobacteriaceae* (*E. coli* and *K. pneumoniae*) isolated at JMC.

- To determine the population structure/clonality of ESBL-producing *E. coli* and *K. pneumoniae* strains isolated at JMC.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study design, study period and Area

A cross-sectional study was conducted at Jimma Medical Center (JMC) located at Jimma, Southwest Ethiopia, from June 2016 G.C to October 2016 G.C. JMC serves as a referral hospital for the large parts of south-west Ethiopia. The hospital also serves as a teaching hospital, medicine and several other health science trainings are being given from lower to higher level of education including nursing, medical laboratory sciences, pharmacy, and midwifery. The hospital also serves as an in-service training center for health professionals on specific courses.

The hospital is staffed with more than 400 regular health care workers including senior physicians, general practitioners, nurses, anesthetists, and laboratory technicians/technologists. Besides, visiting scientists/physicians, medical researchers, and medical students from other countries occasionally visit and participate in service delivery at different clinics of JMC. The hospital has 503 beds and 11 wards for inpatient service with an estimated catchment population of more than 15 million. The service delivery is sub-stratified to 15 specialized clinics/units (e.g. medical, surgical, pediatrics, gynecology). The annual case load was estimated to be 180,000 outpatient visits, 17,000 inpatient admissions, and 12,000 emergency visits in the year 2015.

There are visible challenges in the supply of drugs, appropriate prescription, and rational use of drugs. The overall service delivery at this hospital is often compromised by lack of diagnostic services, lack of consumables for diagnostic setups, awareness of the patients and other facility related factors. The problems of antimicrobial usage or inapproprirate use of antimicrobial has been reported from a study conducted at this hospital. These problems include delay of initiation of effective antimicrobials and excessive use, use without indication or using duplicates of broad spectrum antimicrobials or use of the drug for longer duration than recommended.

2.2 Study population

The study population was recruited from all patients seeking medical care either admitted to the hospital or visiting the hospital as an outpatient on regular working hours. Patients were

selected for this study based on clinical features and clinical diagnosis made by physicians. The participants were recruited for the study when microbiological investigation is needed as part of patient care for treatment and if they fulfill the inclusion criteria. Accordingly, relevant clinical data and appropriate specimens were collected from study participants by experienced nurses working in specified wards for admitted patients and by attending nurses for outpatient participants

2.3 Sample size and sampling technique

The sample size is calculated based on **Slovin's** formula (Ariola, 2006)

$$\mathbf{n = N/1+N.e^2 \quad = 2*10^5/1+2*10^5 *e^2 = 1105}$$

Where: -

n = sample size

N= Total population (annual population of all patients visiting the hospital for infectious diseases) = 200,000

e = precision (margin of error) = 3%

Inclusion criteria

All participants who were volunteers to participate in the study (consent/assent) and provide specimens required for the study were included.

Exclusion Criteria

Participants on antimicrobial treatment for one week were excluded from the study. However, participants not responding to the current antibiotic treatment were included in the study

2.4 Data Collection

Socio-demographic, clinical and laboratory data were recorded and transferred to structured questionnaire prepared for the study (Annex-VII and VIII). Relevant and appropriate clinical specimen was collected from each informed and consented/assented study participant (Annex-I, II, III, IV, V and VI). Moreover, the study participants were informed about their rights to withdraw from the study at any stage of the study based on their request. Study participants were not included in the study when they were not willing to share their personal data and when they were not able to give specimen. The attending physicians participated for patient identification, clinical diagnosis, and collection of clinical data. Experienced nurses transcribed

the clinical data from patient chart to questionnaire and collected specimens. Laboratory technicians were involved in media preparation, specimen processing, culture, and identification of bacterial isolates. The principal investigator carried out overall laboratory tests/investigations, organization, close monitoring/follow of the study and ensuring quality of clinical data and strain collection. The clinical specimens were analyzed for culture and sensitivity at Jimma University Microbiology laboratory. Further sensitivity testing, ESBL screening and molecular characterizations of isolates were done at Karolinska University Hospital, Clinical Microbiology Laboratory, Sweden, by the principal investigator and collaborators. The study was conducted during a period of June 2016 G.C to October 2016G.C.

2.5 Study Variables

Dependent Variables

- Prevalence of ESBL-producing strains
- Antimicrobial susceptibility pattern of ESBL strains
- Molecular characteristics (Molecular classes of ESBLs)/genotypes and clonality of isolates

Independent Variables

Sociodemographic and clinical data of study participants include: age, sex, admission, diagnosis/infection type, current antibiotic usage and type of antibiotic/s used.

2.6 Specimen Collection and Processing

Different clinical specimens were collected from patients based on its relevance to support the clinical diagnosis (pneumonia, sepsis, meningitis, diarrheal illness, wound infection, UTI) and the request was made by examining physician and decision was independent of the study needs**.** Based on the request made by attending physician for microbial investigation different clinical specimens were collected. Accordingly, a total of 1087 specimens were collected from patients having clinical diagnosis of pneumonia, sepsis, diarrheal illness, wound infection UTI and meningitis Clinical specimens collected were as follows:

Sputum (n=267): Sputum specimens were collected from patients suspected with respiratory tract infections and having productive cough. Sputum specimen also collected from children

greater than 15 years of age who were able to produce sputum. Patients were instructed on how to produce sputum and collect in to a wide-mouth, capped and clean sputum collection cup. Then the sputum was evaluated both macroscopically and microscopically to determine the quality. Sputum specimens were accepted for culture only when it was found to containe ≥20-25/polymorphonuclear leukocytes/LPF and <10 epithelial cells/LPF upon Gram staining.

Urine (n=456): Urine specimens were collected from patients clinically diagnosed with urinary tract infection. Non-catheterized patients were instructed how to collect clean catch mid-stream urine into a clean, sterile and wide-mouth container. For catheterized patient's urine specimen were collected from sampling port of the catheter by a nurse. All appropriately collected urine samples were processed and inoculated to media within two hours of collection.

Wound, Exudates, and abscesses from surgical site infection (n=181): Open wound swabs were collected aseptically after the wound surface exudates and contaminants were cleansed off with moistened sterile gauze and sterile normal saline solution. Dressed wounds were cleansed with sterile normal saline after removing the dressing. After cleansing the wound, specimens were collected using sterile cotton swab by rotating the swab with enough pressure. Closed wound abscesses were collected in consultation with experienced nurse or physician.

Stool specimen (n=180): A fresh diarrheic or loose stool specimens were collected before antimicrobial therapy is administered. Patients were given appropriate information how to collect the stool specimen. A clean plastic container and applicator sticks were given for each patient to provide stool specimens. Adult patients were instructed how to collect the stool specimen carefully and nurses collected for children and infants.

Cerebrospinal Fluid (n=3): CSF specimens were obtained from patients with suspected meningitis case by lumbar puncture and collected into sterile container. The LP procedure was performed by the attending physician.

2.7 Culture and Identification of the Bacteria

Sputum, wound swabs, and urine specimens were inoculated on to Blood agar (Oxoid) and MacConkey agar (Oxoid) and incubated 35-37^{0}C for 18-24 hours. Diarrheic/loose stool specimens were inoculated on MacConkey agar, and Selenite F broth for enrichment of *Salmonella* and *Shigella* and incubated for 18hrs at 35-37^{o}C. After overnight incubation any possible growth in Selenite F broth were sub-cultured on MacConkey agar (**Oxoid)** with crystal violet and SSA (**Oxoid)** for isolation of enteric pathogens. After overnight incubation the bacteria any possible growth was sub-cultured to obtain pure colonies and then were identified based on their colony characteristics on their respective media and Gram-staining reaction.

The enteric bacteria isolates were identified based on conventional biochemical tests ((KIA, LIA, Citrate utilization urease production, SIM, and Oxidase) using the WHO guideline for identification of gram-negative bacilli (Basic Laboratory Procedures in Clinical bacteriology, 2nd edition). The species identification was further validated by MALDI-TOF-MS (**MALDI Biotyper RTC**, **Bruker Daltonik GmbH, Bremen, Germany**) at Karolinska University Hospital, Clinical Microbiology Laboratory.

2.8 Matrix Assited Laser Desorption Ionization Time Of Flight Mass Spectroscopy (MALDI-TOF MS):

Mass spectrometry is analytical technique in which chemical compounds are ionized into charged molecules and ratio of their mass to charge (m/z) is measured (Hou *et al*., 2019). A small portion of a pure bacterial colony taken by a tip of tooth pick is prepared by mixing or coating with matrix solution (an energy-absorbent organic compound). After adding the matrix to the sample on a metal otr plastic plate the sample entrapped within the matrix co-crystallizes with matrix crystallizes up on drying. The sample entrapped in the matrix will become ionized when is hit with a laser beam in automated mode. Desorption and ionization with the laser beam generates singly protonated ions from the sample. The protonated ions are then accelerated at a fixed potential, where these separate from each other on the basis of their mass-to-charge ratio (m/z). The charged analytes are then detected and measured using different types of mass analyzers like quadrupole mass analyzers, ion trap analyzers, time of flight

(TOF) analyzers etc. For microbiological applications mainly TOF mass analyzers are used (Singhal *et al*., 2015).

During MALDI-TOF analysis, the m/z ratio of an ion is measured by determining the time required for it to travel the length of the flight tube. A few TOF analyzers incorporate an ion mirror at the rear end of the flight tube, which serves to reflect back ions through the flight tube to a detector. Based on the TOF information, a characteristic spectrum called peptide mass fingerprint (PMF) is generated for each species of a bacteria (Lartigue, 2013).

2.9 Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing was performed for all *Enterobacteriaceae*, *P. aeruginosa* and *Acinetobacter spp.* isolated from clinical specimens using different antimicrobial agents according to a recommendation by EUCAST disk diffusion AST guidelines (EUCAST 2016).

The following antimicrobial agents were used for susceptibility testing of *Enterobacteriacea*e strains: Piperacillin-tazobactam (30-6µg), Cefotaxime (5µg), Ceftriaxone (30µg), Ceftazidime (10µg), Meropenem (10µg), Ertapenem (10µg), Imipenem (10µg) Gentamicin (10µg), Amikacin (30µg), Ciprofloxacin (5µg) and Trimethoprim-sulfamethoxazole (25µg) (Oxoid).

For *Pseudomonas spp.*, Ceftazidime (10µg), Piperacillin-Tazobactam (25µg), Gentamicin (10µg), Amikacin (30µg), Ciprofloxacin (5µg), Imipenem (10µg) and Meropenem (10µg) were used. For antimicrobial testing of *Acinetobacter spp*., Gentamicin (10µg), Amikacin (30µg), Ciprofloxacin(5µg), Trimethoprim/sulfamethoxazole (25µg), Imipenem (10µg) and Meropenem (10µg) discs were used.

Briefly, a standardized suspension of each isolates were prepared using normal saline and matched with the turbidity standard McFarland 0.5. The standardized suspension was streaked in to Muller-Hinton Agar (Oxoid) using sterile cotton tip applicator stick and allowed to dry. After that the antibiotic discs were placed on the medium and incubated at 35-37°C for 16-18 hours.. After the appropriate incubation time, the zones of inhibition were measured using caliper and interpreted as sensitive, intermediate and resistant according to EUCAST disk diffusion AST guidelines (EUCAST 2016).

ESBL screening

In the study site, all bacterial strains isolated were screened for decreased susceptibility to ceftriaxone (30µg), cefotaxime (30µg), ceftazidime (30µg) and amoxiclavulanic acid antibiotic disks **(Oxoid**) by Kirby-Bauer disk diffusion method (Hudzicki, 2009). Any decreased zone of inhibition around these disks were measured and interpreted according to CLSI guidelines for interpretation for ESBL phenotype screening (CLSI 2016). Decreased susceptibility to these disks were considered as potential ESBL strain and selected for further phenotypic confirmatory test. Furthermore, additional ESBL screening was performed at Karolinska University Hospital clinical microbiology laboratory with cefotaxime (5µg) and ceftazidime (10µg) disks, according to EUCAST recommendation.

Phenotypic ESBL confirmatory test

In the study site, ESBL phenotypic confirmatory tests were performed for strains that showed decreased susceptibility or resistance to ceftriaxone (30µg), cefotaxime (30µg) and/or ceftazidime. The suspected strains were inoculated onto Muller-Hinton agar (Oxoid). After that antimicrobial disks cefotaxime (30µg), ceftazidime (30µg) and ceftriaxone (30µg) were placed 20-30 mm centre to centre together with amoxicillin-clavulanic acid (20/10) placed at the centre. An increase in zone of inhibition of either of the third-generation cephalosporins towards clavulanic acid was interpreted as phenotypically confirmed ESBL-producing strain.

2.10 Strains Storage and Transportation

All strains isolated were stored in a 20% glycerol/tryptone-soya broth at -80^{0}C freezer. Then shipped in dry ice to Karolinska University Hospital, Division of Clinical Microbiology for validation (species identification, and antimicrobial susceptibility testing), and molecular characterizations of ESBL strains. Material transfer agreement was signed between host institution and collaborative institution before shipment of the isolates.

2.11 Molecular Characterizations

The whole genome sequencing was performed using an Illumina HiSeq 2500 2x150bp paired end sequencing protocol at *Science for life laboratory*, Stockholm, Sweden. A total of 249 strains of *E. coli* (n=140) and *Klebseilla spp*. (n=109) were inoculated onto CLED medium and

incubated at 37^0 C. After overnight incubation 2-5 pure colonies were taken with inoculating loop and allowed to lyse in BSA (0.5mg/ml) in PBS (PH =7.4). The lysate was dispensed in to 96 well plates for DNA extraction. Then the DNA was extracted by automated *MagnaPure96* (Roche, Life sciences, Sweden) DNA extraction system. The concentration of DNA was measured by using Qubit 3.0 (**Thermo scientific, Malasiya**). The DNA extraction, quantification and preparation were made based on the sample submission guidelines from the sequencing center. Libraries were generated using Nextera DNA library preparation kit (Illumina).

Genome sequencing and assembly: Sequence reads (.fastq files) were received together with report on the quality of the sequencing, about fraction mapped, fraction duplicate, coverage, and total number of reads. The report indicated a very good quality of sequencing run. We have used two pipelines for assembly the Enterobase for *E. coli* strains and Center for Genomic Epidemiology for both *E. coli* and *K. pneumoniae*.

Genome assembly was performed by using SPAdes (version 3.9) genome assembly tool and also embedded within two different pipelines/online databases for genomic analysis of *Enterobacteriaceae* and other bacterial strains including gram-positive and gram-negative. The first data base (https://cge.cbs.dtu.dk/services/SPAdes/) for both *E. coli* and *K. pneumoniae* is at center for genomic epidemiology which is hosted by the Technical University of Denmark (DTU).

The second database Enterobase (http://enterobase.warwick.ac.uk/species/index/ecoli) used only for *E. coli*, it is a global database hosted by Warwick Medical School, England. In both databases, the assembly pipelines (SPAdes) operate with concordant assembly parameters.

SNP based phylogenetic analysis: Variant calling, mapping and *de novo* assembly was done using CLC Assembly Cell Version: 4.4.2.133896 **(**Qiagen Bioinformatics). Minimum coverage was set to 10 and the SNP ratio cut-off for SNP support was set to 90%. Gubbins version 2.3.1, with standard settings, was used to remove SNPs from recombinant regions. Maximum likelihood tree was inferred with FastTree/2.1.8 using GTR nucleotide substitution

model and bootstrapping (1,000 replicates). Visualisation of trees and metadata was done using iTOL (https://itol.embl.de/).

Resistome (acquired resistance genes) for *E. coli* and *Klebseilla. spp*. was identified and extracted from *ResFinder-3.0***,** web-tool to retrieve mainly acquired resistance genetic determinants among species of *Enterobacteriaceae*. The tool is hosted at Center for Genomic Epidemiology (CGE) (https://cge.cbs.dtu.dk/services/ResFinder/).

Virulence genes (*E. coli)* were extracted from the CGE database using the bioinformatics tool called *VirulenceFinder 1*.5, (https://cge.cbs.dtu.dk/services/VirulenceFinder/).

Plasmid replicon types were determined for both *E. coli* and *Klebseilla spp.* by using PlasmidFinder ver.1.3 **(**https://cge.cbs.dtu.dk/services/PlasmidFinder/), a bioinformatics tool to identify plasmids commonly associated with species that belong to *Enterobacteriaceae*.

MLST typing:- for both *E.coli* and *Klebsiella spp.,* web-tool designed for all ord.nov. *Enterobacterales* (MLSTver.1.8) hosted at (https://cge.cbs.dtu.dk/services/MLST/) was used. Serotyping/serotype prediction of *E. coli* strains was performed by **SerotypeFinder1.1**, a whole genome based prediction tool **(**https://cge.cbs.dtu.dk/services/SerotypeFinder/**).** This tool is also nested within same database, Center for Genomic Epidemiology.

Capsular and LPS typing of *K. pneumoniae* strains, a recently established database that uses whole genome sequence data for prediction of capsular type and LPS types called Kaptive: (http://kaptive.holtlab.net/) was used. Kaptive is nested within Holt Lab, hosted at University of Melbourne, Australia. The database relies on the capsular locus and provides a nomenclature scheme and revealed over 150 different capsular types. Kaptive is a tool for *Klebsiella* locus typing and variant evaluation. It takes one or more pre-assembled genomes and for each finds the best matching locus from a reference database. References for known K-locus (capsule) and O-loci (lipopolysaccharide) are available in Kaptive's web interface.

In all the web-tools used and mentioned above, we defined or considered the presence of a genetic determinant for a trait we are looking for only when the query sequence is 100% matched both in base sequence and sequence segment length. In this study, the default parameters of each tool were used accordingly.

Enterobase: another web-based whole genome data analysis database for *E. coli* and few other strains(https://enterobase.warwick.ac.uk/). Enterobase is stricter than Center for Genomic Epidemiology for analysis and produce report on quality of reads, and success of assembly. We used the default parameters for assembly and genomic analysis of *E. coli* strains for resistance genes, plasmid typing, MLST, and serotype prediction.

Gubbins: another bioinformatic tool, was used for studying population structure. SNP based phylogenetic analysis was performed for both *E. coli* and *K. pneumoniae* strains.

2.12 Quality Control

Standard and conventional guidelines were used for isolation and identification of strains. Culture media **(Himedia, India)**, biochemical tests (**Oxoid)** reagents, chemicals, antimicrobial discs (**Oxoid)** and all other necessary supplies were obtained from sources of known standard or quality. Each of the culture media were prepared according to the instruction on the bottle and checked for sterility by incubating 5% of each batch of preparation. Furthermore, strains were also validated using MALDI-TOF for strain identification and antimicrobial susceptibility testing at Karolinska University Hospital clinical microbiology laboratory.

Control strains for isolation and identification of gram-negative bacilli, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used in general. For β-lactamase test mainly among *Enterobacteriaceae* species, *E. coli* ATCC 35218 were used to optimize and ensure correct isolation, identification, and antimicrobial susceptibility of strains.

About 10% of clinical data collected on questionnaire were cross-checked with patients back for accuracy and validity. All data were checked for validity, clarity, and completeness and entered into Statistical software.

2.13 Data Processing and Analysis

Both clinical and laboratory data were cleaned, checked for completeness, and entered to statistical software (IBM.SPSS. statistics. v21. for windows).

Genome sequence data were analyzed by different web-based NGS data analysis pipelines. There were different data analysis databases/pipelines used to analyze the genome sequences based on the scope and relevance of the databases to the objectives of the study.

2.14 Ethical Consideration

The PhD research project was approved and ethically cleared in the following order by:

- Department Ethical Review Committee (DERC) of the Microbiology, Immunology and Parasitology (DMIP), CHS, AAU.
- Institutional Review Board (AAU-IRB) of the Colleges of Health Sciences, Addis Ababa University (ref no. 002/16)
- AHRI-ALERT Ethics Review Committee (AAERC) (ref no. PO07/16)
- National Research Ethics Review Committee (NERC), Ministry of Science and Technology (Ref. no 3-10/150/2016)

In addition, the protocol was approved and permission was obtained from Jimma University Teaching Hospital, south west Ethiopia before the commencement of the project. Written informed consent was obtained from each adult study participants (Annex II). For participants less than 18 years old, written informed consent was obtained from their parents/guardian (Annex IV). In addition, assent was also obtained for study participants between 12 and 17 years old (Annex VI). The aim of the study, its significance, confidentiality, participation right, procedure and associated risks were explained through an information sheet (Annex I, II and V). Culture and sensitivity results were promptly reported to the attending physician for patient care. Moreover, findings of the present study will be communicated with responsible bodies for prevention and control of AMR and will be published on National and International Journals to disseminate information.

CHAPTER 3: RESULTS

3.1 Characteristics of study population

A total of 1105 study participants were recruited for data collection and 1087 study participants were considered for analysis after the data was checked for completeness and edited for uniformity. Of these study participants 41.1% were female and 58.9% were male (**Table:3.1**). The age of the study participants ranges from 02 months to 90 years, the mean age was 32 and the median age was 30 with standard deviation of 20.2. Study subjects were recruited from both inpatient and outpatient departments at four major units of the hospital (Medical, Surgical, Pediatric and ICU).

Table 3.1 Age-Sex distribution of study participants at JMC, Ethiopia.

			Sex		**Total**
			Female	**Male**	
Age category	<1	Count	5	6	11
		%	45.5%	54.5%	100.0%
	>=1&<5	Count	40	66	106
		%	37.7%	62.3%	100.0%
	>=5&<15	Count	38	67	105
		%	36.2%	63.8%	100.0%
	>=15	Count	364	501	865
		%	42.1%	57.9%	100.0%
Total		**Count**	**447**	**640**	**1087**
		%	**41.1%**	**58.9%**	**100.0%**

Single (non-repeat) clinical specimens were collected from each study participant based on the clinical diagnosis and recommended standard practice of microbiological investigation and urine, sputum, wound swab, and stool were collected form patients clinically diagnosed with urinary tract infections, pneumonia, wound infections, and diarrhea respectively.

The enrolment of the study subjects to the study was mainly based on active infection on-going during data collection to one of the above-mentioned infections. Some of these patients had other underlying chronic diseases including but not limited to diabetes, HIV, tuberculosis, heart diseases, and different neoplastic diseases. Thus, patients with chronic illness were recruited

for the study because of super-imposed infections. From pediatric study participants, most of them were primarily admitted to the hospital for severe acute malnutrition (SAM), and these patients were recruited when they have developed diarrhea.

Table 3.2: Types and frequency of specimens collected from patients at JMC, 2016.

Specimen			**Admitted/Outpatient**		**Total**
			Admitted	Outpatient	
	Sputum (24.5%)	Count	158	109	267
		%	59.2%	40.8%	100.0%
	Wound swab (16.7%)	Count	157	24	181
		%	86.7%	13.3%	100.0%
	Stool (16.6%)	Count	155	25	180
		%	86.1%	13.9%	100.0%
	Urine (41.9%)	Count	329	127	456
		%	72.1%	27.9%	100.0%
	CSF (0.3%)	Count	3	0	3
		%	100.0%	0.0%	100.0%
		Count	**802**	**285**	**1087**
Total		**%**	**73.8%**	**26.2%**	**100.0%**

Urine microbiologic examination was the most frequently requested test followed by sputum. Most of study population 73.8% were admitted patients, and 26.2% were outpatients (**Table 3.2**).

The types of disease conditions observed during the study were so divers and several specific cases attended at the hospital might be difficult to summarize each case one by one. For simplicity of understanding patient cases determined/diagnosed at JMC under this study were summarized into five different categories (**Table 3.3**)

Table 3.3. Frequency and type of diagnosis among study participants at JMC

Diagnosis	Frequency	%	Cumulative (%)
[&]UTI	456	42.0	42.0
Pneumonia	245	22.5	64.5
Diarrhea	181	16.7	81.1
Wound Infection	179	16.5	97.6
[*]COPD	26	2.4	100.0
Total	**1087**	**100.0**	

[&]UTI = Urinary Tract Infection, [*]COPD= Chronic Obstructive Pulmonary Disease

In general, a total of 642 bacterial strains were collected by conventional bacteriologic tests at species level and some at genus level (WHO Manual of Bacteriology 2nd edition). The bacterial profile isolated from all the units in the hospital seem to have similar pattern. *E. coli* the most common bacterial isolates in all the four units.

As described in the method section, further characterization of the strains by MALDI-TOF at Karolinska University Hospital, Department of Clinical Microbiology was used to validate the conventional species identification at study site. The use of MALDI-TOF for screening showed more diversity of the strain collection. Hence, more than 25 different species of bacteria were identified. Nevertheless, the most common strains isolated were *E. coli*, *K. pneumoniae*, *Enterobacter spp. P. aeruginosa*, and *Acinetobacter spp.,* and also the highest prevalence of ESBL strains were identified among *E. coli* and *K. pneumoniae* followed by non-fermenters *Acinetobacter* and *Pseudomonas spp*. (see **Table 3.4**).

Table 3.4: The rate of ESBL in each types of strains isolated at different units of JMC

S. No	Species isolated	Unit at which the strain was isolated									
		Medical		Surgical		Pediatric		ICU		Total	
		# of strains isolated	# of ESBL(%)	# of strains isolated	# of ESBL(%)	# of strains isolated	# of ESBL(%)	# of strains isolated	# of ESBL(%)	# of strains isolated(%)	# of ESBL(%)
1	*E. coli*	81	41(50.6)	76	51(67.1%)	102	50(49)	3	2(66.6)	262(40.8)	144(54.9)
2	*Klebsiella spp.*	50	30(60)	38	32(84.2)	52	45(86.5)	6	4(66.7)	146(22.7)	111(76.0)
3	*Enterobacter spp.*	27	17(62.9)	25	21(84)	9	4(44.4)	1	1(100)	62(9.6)	43(69.3)
4	*P. aeruginosa*	8	5(62.2)	27	6(22.2)	2	-	5	3(60)	42(6.5)	14(33.3)
5	*Acinetobacter spp.*	7	3(42.8)	33	21(63.3)	2	1(50)	3	3(100)	45(7)	28(62.2)
6	*Citrobacter spp.*	4	2(50)	14	11(78)	4	2(50)	1	1(100)	23(3.5)	16(69.5)
7	*Providencia spp.*	3	1(33.3)	14	11(78)	3	2(66.6)	-	-	20(3.1)	14(70)
8	*Proteus spp.*	5	-	11	2(18.1)	1	1(100)	-	-	17(2.6)	3(17.6)
9	*M. morganii*	3	2(66.6)	6	4(66.7)	1	1(1000	1	1(100)	11(1.7)	8(72.7)
10	Others*	5	1(20)	7	4(57.1)	1	-	1	-	14(2.1)	5(35.7)
Total		**193**	**102(52.8%)**	**251**	**163(64.9)**	**177**	**106(59.8)**	**21**	**15(71.4)**	**642(100)**	**386(60.1)**

Note:

1. *Klebseilla spp.* (*K. pneumoniae, K. variicola, K. oxytoca*), *Enterobacter spp.* (*E. cloacae, E. kobei, E. asburiae*), *Acinetobacter spp.* (*A. baumanii, A. baylayi, A. junii, A. calcoaceticus, A. lowfii, A. schindleri, and A. radioresistens*), *Citrobacter spp.*(*C. freundii, C.sedalkii, C.braakii*), *Providncia spp.* (*P. stuartii, and P. retgerii*), *Proteus spp.* (*P. mirabilis, P. hauseri,and P. penneri)*.
2. Others* (*Leclercia kerestersii* (02), *Serratia rubidea* (01), *Serratia marscecens*(01), *Camomonas kerestersii* (01), *S. maltophila* (01), *Kerestersia gyiorum* (01), *K. ascorbate* (01), *Salmonella newington* (01), *Roultella ornithinilytica* (01), *Aeromonas hydrophilia* (01) *Kasaconia cowanii* (01), and *Ponteo septica* (01)).
3. Acinetobacter spp. were tested for (meropenem and imipenem), and hence, these data show non-susceptibility of *Acinetobacter spp.* to carbapenems.

The most prevalent isolates from the overall collection were *E. coli* (40.8%) and *K. pneumoniae* (22.7). Overall prevalence of ESBL was higher among all strains isolated from patients at surgical unit. *E.coli* and *K. pneumoniae* were the predominant strains isolated from these patients at all units. With regard to species related prevalence of ESBL, *Klebsiella spp.*, *Enterobacter spp.*, and *Acinetobacter spp.* were strains with higher prevalence of ESBL. However, the total number of *Enterobacter spp.* and *Acinetobacter spp.* were significantly smaller than the number of both *E.coli* and *Klebsiella spp.* in this study.

3.1.1 Most commonly isolated strains of Gram-negative bacilli

From 642 bacterial strains that belong to several other species and genus, the most commonly isolated strains were *E. coli* and *K. pneumoniae* from *Enterobacterales, P. aeruginosa spp.* and *Acinetobacter spp*. from non-fermenters (**Figure 3.1**).

Other strains also commonly isolated from clinical setups include:- *Enterobacter spp*. *Proteus spp., Citrobacter spp.*, *Aeromonas spp., Leclercia spp.,*were also identified. Several other species of Gram-negative bacteria rarely reported from clinical specimens, *Leclerecia adecarboxylata*, *Comamonas kerstersii*, *Raoultella ornithinolytica, Kerestersia gyiorum*, and *Kluvera ascorbate* were also isolated. Relative proportion of clinically important strains isolated were presented in **Figure 3.1**.

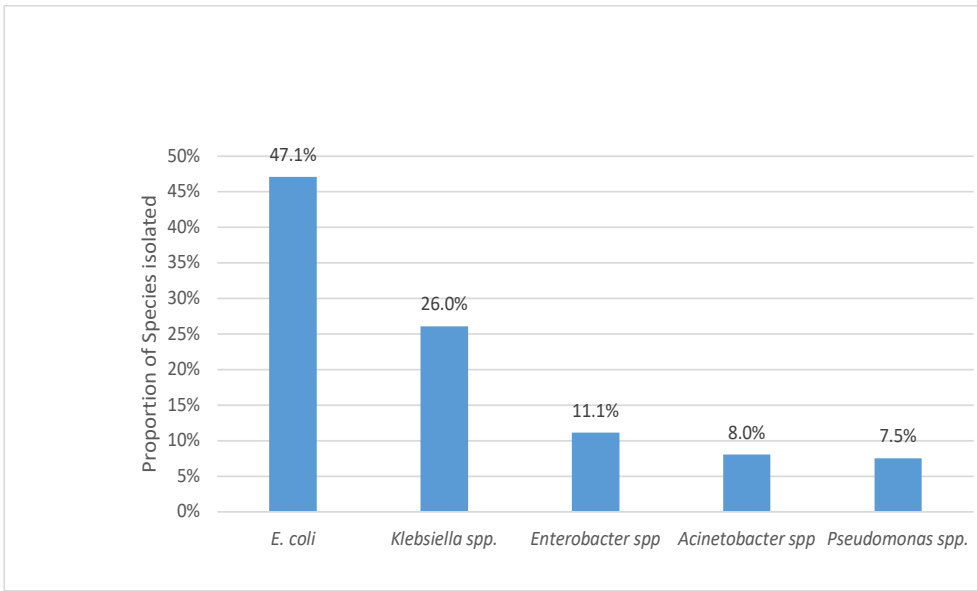

Figure 3.1: Relative proportion of most frequently isolated species

Socio-demogrphic factors associated to rate of ESBLs

Some possible risk factors for either acquisition of resistance genes and/or acquisition of resistant strains were studied. The rate of ESBL was higher from admitted patients (82.2%, 303/369) as compared to rate of ESBL among outpatients (17.8%, 66/369) (p-value = 0.000). The rate of ESBL among patients with chronic disease was (30.8%, 271/703) and among patients with no-chronic disease was (25.5%, 98/384)(p-value = 0.000). Similarly, a higher rate of ESBL was observed among study participants in the lower age category, < 1year (90.9%, 10/11), >=1 &<5 years (78.3%, 83/106), >=5&<15 years (45.7%, 48/105) and >15 (26.4%, 228/863) (p-value = 0.000). Rate of ESBL were higher among diarrhea samples (90.6%, 164,181) (p-value = 0.000). Overall highest rate of ESBL (64.9%, 163/386) was observed from strains isolated from specimen collected at surgical unit (p-value =0.000).

With regard to antimicriobial consumption: 1) if the patient is currently on antimicrobial therapy, 2) previous antimicrobial usage (in the last three months) was recorded from the patient chart for admitted patients and verbal report for outpatient study subjects. Antimicrobial usage as a risk factor was studied and study subjects were interviewed for any antimicrobial that might have been taken in the last three months before enrolment to the study. Antibiotics reported to have been used includes, ampicillin, amoxicillin, amoxicillin-clavulanicacid (augumentin), ceftriaxone, gentamicin, ciprofloxacin, metronidazole, chloramphenicol, clindamycin, cotrimoxazole, doxycycline, cloxacillin, norfloxacin, vancomycin, anti-tuberculosis and anti-retroviral drugs.

Overall, 62.8%(684/1087) of study subjects were reportedly on antimicrobial therapy during the study. Moreover, 39.5% (43/1087) of these patients were currently on ceftriaxone treatment as monotherapy or combined with other drugs. Next to cephalosporins, penicillins (19.5%), metronidazole (11.7%), phenicols (5.8%), aminoglycosides (3.8%), and fluoroquinolones (2.7%) were the most common antimicrobials prescribed at the hospital during the study period. However, any recent antibiotic use was not statically associated to increased rate of ESBL in general sense (p-value = 0.72). Also, there was no statistically significant difference in rate of ESBL between male and female particpants (p-value=0.685).

3.2 Phenotypic and molecular features of *E. coli* strains

3.2.1 Phenotypic characteristics of *E. coli* strains

E. coli (n=262) were collected from the four units of the hospital (ICU, pediatric, medical, and surgical). The prevalence of ESBL-producing strains among *E. coli* was 54.9% **(Table 3.5)**. The ESBL-strains were also resistant to other classes of antimicrobials at a high rate of resitance including: gentamicin (62.4%), ciprofloxacin (90%), trimethoprim/sulfamethoxazole (93%) and piperacillin-tazobactam (79.4%). Furthermore, some of these strains are non-susceptible to meropenem (screening), and these strains accounted for 5.4% of ESBL-producing *E. coli* **(Table 3.6).**

Table 3.5: Prevalence of ESBL-producing *E. coli* strains JMC, 2016.

ESBL-phenotype	Frequency
Yes	**144(54.9%)**
No	118(45.1%)
Grand Total	**262(100%)**

Table 3-6: Prevalence of meropenem non-susceptible *E. coli* strains at JUTH, 2016**.**

Meropenem non-susceptible	Frequency
Yes	**14(5.4%)**
No	249(94.6%)
Grand Total	**262(100%)**

3.2.2 MDR strains and antibiogram

All ESBL strains (n=144) were tested for ten different drugs according to the recommendation of EUCAST-2016 guideline for antimicrobial susceptibility testing for *Enterobacteriaceae*. Strains were variably resistant to both drugs from the same class of antimicrobials and to drugs from different classes of antimicrobials. All ESBL-producing strains were resistant to at least three different classes of drugs and a maximum of six classes of antimicrobials (nine different drugs) (see **Figure:3-2**).

Most of the strains were multiple/multidrug resistant. More than 80% of these strains were resistant to at least four classes of antimicrobials. Furthermore, the genetic background of these

strains and the genetic determinants of antimicrobial resistance is discussed elsewhere in this document. The phenotypic antibiogram is depicted below (**Table:3.7**)

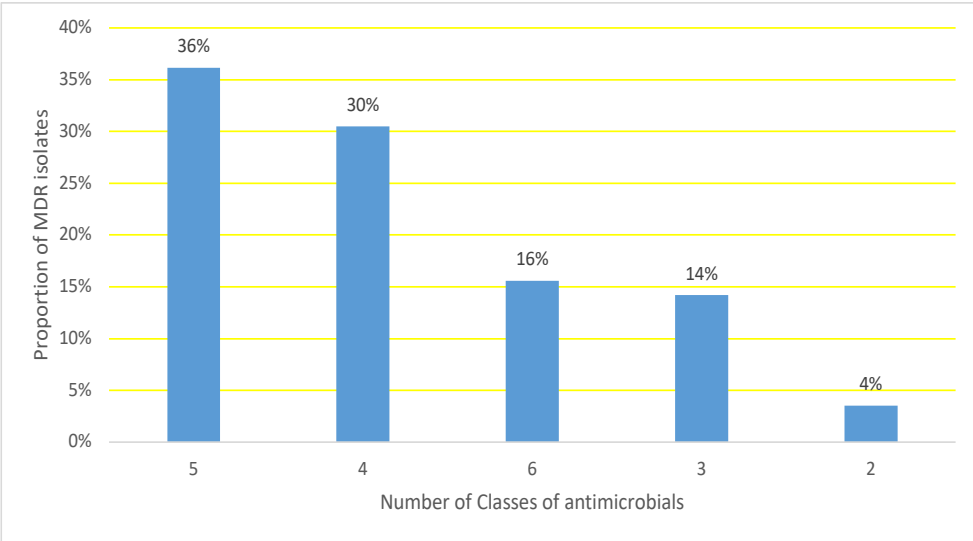

Figure 3.2: Rate of MDR among clinical strains at JMC, 2016.

Multiple drug resistance both within same class of antimicrobials and between different classes of antimicrobials were determined. Nine different antimicrobials that fall into six main classes of antimicrobials and frequently prescribed at the study site were selected. The representative drugs tested for these classes of antimicrobials were cephalosporins (cefotaxime, ceftazidime, and ceftriaxone), aminoglycosides, (amikacin and gentamicin), fluoroquinolones (ciprofloxacin), trimethoprim-sulfamethoxazole, extended-spectrum penicillin (piperacillin-tazobactam), and carbapenems (meropenem, ertapenem, and imipenem).

Table 3.7: Antibiogram of ESBL-producing *E. coli* strains isolated from JMC

# Strains (%)	Antibiogram of ESBL strains of *E. coli* (%)	#of drugs	#Classes of drugs
5(4%)	*CTX-PTZ-CAZ (10%)*	3	2
	CTX-CAZ-SXT (40%)		
	CTX-CIP-CAZ (40%)		
20(14%)	*CTX-CIP-SXT (5%)*	3	3
	CTX-CAZ-CIP-SXT (50%)	4	
	CTX-CAZ-PTZ-CIP (15%)		
	CTX-CAZ-PTZ-SXT (30%)		
43(30%)	***CTX-CAZ-PTZ-CIP-SXT (46.5%)***	5	4
	CTX-CAZ-CIP-SXT-CN (30.2%)		
	CTX-CAZ-PTZ-CIP-CN (6.9%)		
	CTX-CAZ-PTZ-SXT-ETP (2.3%)		
	CTX- CAZ-PTZ-SXT-CN (9.3%)		
	CTX-CAZ-PTZ-SXT-CN-AK (4.6%)	6	
51(36%)	***CTX- CAZ-PTZ-CIP -SXT-CN (72.5%)***	6	5
	CTX-CAZ-PTZ-CIP- SXT-ETP (5.8%)		
	CTX-CAZ-PTZ- SXT-CN-AK (11.7%)		
	CTX-CAZ-PTZ-CIP-SXT-ETP-MEM (3.9%)		
	CTX-CAZ-PTZ-CIP- SXT-MEM-CN (3.9%)		
	CTX-CAZ-PTZ-CIP-SXT-AK (1.9%)		
22(16%)	***CTX-CAZ-PTZ-CIP-SXT-ETP-CN (36.3%)***	7	6
	CTX-CAZ-PTZ-CIP-SXT-MEM-CN (4.5%)		
	CTX-CAZ-PTZ-CIP-SXT-ETP-MEM-CN (36.3%)	8	
	CTX-PTZ-CIP-CAZ-SXT-ETP-CN-AK (9%)		
	CTX-PTZ-CIP-CAZ-SXT-ETP-IPM-MEM-CN (4.5%)	9	
	CTX-PTZ-CIP-CAZ-SXT-ETP-MEM-CN-AK (9%)		

CTX: Cefotaxime, PTZ: Piperacillin-Tazobactam, CIP: Ciprofloxacin, CAZ: Ceftazidime, AK: Amikacin
SXT: sulfamethoxazole, ETP: Ertapenem, MEM: Meropenem, IPM: Imipenem, CN: Gentamycin

E. coli strains were tested for susceptibility to combination of drugs either from the same class or different classes. The above table shows that all strains were multiple drug resistant, and (i) the first-column (strains)- shows that the overall proportion of *E. coli* strains in that category (the number of classes antimicrobials that the strains are resistant for), (ii) second-column (antibiogram of ESBL strains) shows that type of each antibiotic and specific combination with its type of drugs and relative proportion in that specific category (iii) third-column (#drugs)- shows number of drugs these strains were resistant to regardless of whether the antimicrobial agents are from the same class or not, (iv) fourth-column (# of classes of antimicrobials)- shows for how many classes of drugs these *E. coli* strains were resistant for.

3.2.3 Molecular characteristics *E. coli* strains

All *E. coli* strains phenotypically confirmed as ESBL-producing were selected for whole genome sequencing (Next Generation Sequencing) and genomic DNA was extracted for (n=141) *E. coli* strains. Then these strains were sequenced at *SciLifeLab* on Illumna (HiSeq 2500) sequencing platform. Whole genome data was obtained as raw reads (.fastq format), then assembled, and the assembled genomic data was used to query the ***ResFinder*** database for acquired resistance genes and other molecular features like virulence genes, serotypes, MLST, and plasmid replicon typing.

3.2.4 Resistance genes:

Multiple ESBL-genes were detected from most of the strains, several other resistance genes for non-ESBL related to other classes of antibiotics commonly prescribed drugs for infections caused by *E. coli* were detected. All strains were defined with a particular resistance genes for ESBL, however, some of the strains encoded multiple ESBL-genes. These strains encode also genetic determinants of resistance to other classes of antimicrobials. In addition to encoding multiple ESBL- and non-ESBL-genes, some of the strains also contain several copies of a given resistance gene. Furthermore, multiple variants of genetic determinants of rsistance for a given class of antimicrobials were also identified. (**Table:3.8**).

These antimicrobial agents and their corresponding genetic determinants of resistance include-trimethoprim (*dfrA*), sulfonamides (*sul1*, *sul2*, *sul3*), tetracycline (*tetA*, *tetB*, *tetD*), phenicols (*catA1*, *catB3*, *catB4*) and aminoglycosides (*aac*, *aad*, *str*). Co-existence of aminoglycosides resistance genetic factors and ESBL-genetic determinants, mainly with the prevalent $bla_{\text{CTX-M-15}}$ was high. The *aac(6')-Ib-cr* resistance gene, which can render strains the ability to resist both aminoglycosides and floroquinolone and other two (*aadA5* and a*adA2*) aminoglycosides resistance genes were associated to $bla_{\text{CTX-M-15}}$ (P-value =0.006, 0.028 and 0.000 respectively) (**Table:3.9**).

Table 3.8: **Distribution of resistance genes among *E. coli* strains**

S. No.	Class of antimicrobials	Sub class genes	Genes detected	Frequency (%)
1.	Aminoglycosides	Acetylation genes	*aac(3)-IIa*	2(1.4)
			aac(3)-IId	2(1.4)
			aac(6')-IIc	1(0.7)
		Aminoglycosides and Floroquinolone	***aac (6') Ib-cr**	**87(62.6)**
		Adenylation	*aadA1*	8(5.7)
			aadA2	11(7.9)
			aadA5	75(54)
			aadB	3(2.1)
		Streptomycin	***strA**	**71(51.0)**
			strB	55(39.5)
		Phosphorylation	*aph(3')-Ia*	1(0.7)
2.	β-lactams	CMY	bla_{CMY-2}	17(12.2)
			bla_{CMY-42}	2(1.4)
		CTX-M	$bla_{CTX-M-11}$	4(2.8)
			$bla_{CTX-M-14}$	6(4.3)
			***$bla_{CTX-M-15}$**	**123(88.4)**
			$bla_{CTX-M-27}$	3(2.1)
			$bla_{CTX-M-55}$	1(0.7)
		OXA	***bla_{OXA-1}**	**88(63.3)**
			bla_{OXA-10}	2(1.4)
			bla_{OXA-66}	1(0.7)
		TEM	bla_{TEM-1B}	75(53.9)
		SHV	bla_{SHV-12}	1(0.7)
3.	Carbapenems	NDM	bla_{NDM-1}	1(0.7)
4.	Phenicols		*catA1*	6(4.3)
			***catB3**	**79(56.8)**
			catB4	2(1.4)
5.	Trimethoprim		*dfrA1*	13(9.3)
			dfrA12	11(7.9)
			***dfrA17**	**81(58.2)**
			dfrA27	3(2.1)
			dfrA5	7(4.9)
			dfrA7	5(3.5)
			dfrA8	2(1.4)
			dfrB4	1(0.7)
6.	Fosfomycin		*fosA*	1(0.7)
7.	Macrolides		***mph(A)**	**94(67.6)**
			qepA	1(0.7)
8.	Fluoroquinolones		*QnrB1*	1(0.7)
			QnrB4	1(0.7)
			QnrB6	1(0.7)
			***QnrS1**	**8(5.6)**

9.	Tetracycline	*tet(A)*	49(35.2)
		tet(B)	**62(44.6)**
		tet(D)	1(0.7)
10.	Sulfonamides	**sul1*	**95(68.3)**
		Sul2	80(57.5)
		sul3	1(0.7)
11.	Rifampicin	*arr2*	1(0.7)
		**arr3*	**3(2.1)**
Total			**139(100)**

*Genes with higher proportion in their category

β-lactamase/ESBL genes:

***bla*CTX-M genes**: *bla*CTX-M genes were prevalent among these strains (*bla*CTX-M-3, *bla*CTX-M-9, *bla*CTX-M-11, *bla*CTX-M-14, *bla*CTX-M-15, *bla*CTX-M-27, and *bla*CTX-M-55). The *bla*CTX-M-15 was most prevalent *bla*CTX-M gene, and identified in 123/139 (88.5%) of strains. Only one of these strains harbor more than one *bla*CTX-M genes.

***bla*TEM genes**: *bla*TEM-1B is the only class of *bla*TEM genes identified among this collection of *E. coli* and it was found in 75/139(53.9%) strains.

***bla*OXA genes**: among *bla*OXA genes *bla*OXA-1, *bla*OXA-10, and *bla*OXA-66 were variants of *bla*OXA identified. *bla*OXY-1-2 were rarely identified and the overall prevalence of *bla*OXA genes was 88/139(66.3%). Other *bla*OXA-type genes were detected in small number of strains.

***bla*CMY genes:** *bla*CMY-2 and *bla*CMY-42 were two *bla*CMY genes detected, and these genes are relatively lower proportion of β-lactamase genes in this study (19 strains were carrying these genes, of which 17/19 (89.5%) were *bla*CMY-2 and 2/17(10.5%) were *bla*CMY-42). All strains that harboured *bla*CMY genes also carried *bla*CTX-M-15 and other β-lactamases.

Carbapenem resistance: all ESBL-producing *E. coli* strains were tested for carbapenem resistance. Phenotypic analysis revealed some strains were non-susceptible to carbapenem, however, genetic determinant of carbapenemase meditaed resistance was identified in only two strain (the class-B metalo-β-lactamase, *bla*NDM-1, and the class-D OXA-51-like, *bla*OXA-66).

Aminoglycosides resistance: the resistance genes for aminoglycosides were also determined in the same manner. Most of them plasmid mediated resistance genes, and several variants of related group of sub-class genes.

The correspondening factors encoded by these resistant genes include aminoglycosides modifying enzymes:- acetylating enzymes -(*aac (6')-Ib-cr*, *aac(3)-IIa*, *aac (3)-IId*, *aac(6')-IIc*), adenylatingenzymes-(*aadA1*, *aadA2*, *aadA5*, aadB), phosphorylating enzymes-(*aph(3')-Ia*), and phospho-transferase enzymes -(*strA*, *strB*). The plasmid mediated *aac(6')-Ib-cr* gene which encode resitance for both fluoroquinolone and aminoglycosides account for 87/139 (62.5%). Among *bla*$_{\text{CTX-M-15}}$ strains 82/123(66.5%) also carry *aac(6')-Ib-cr* gene. The *aadA2* and *aac(6')-Ib-cr* genes were identified frequently with *bla*$_{\text{CTX-M-15}}$ ESBL-genes (**Table:3-9**).

Table 3.9: Relationship between *bla*$_{\text{CTX-M-15}}$ genes and Aminoglycosides modifying enzymes

AGMG*		***bla*$_{\text{CTX-M15}}$ genes**			**P-value**
		No (%)	**Yes (%)**	**Total (%)**	0.006
aac(6')-Ib-cr	No	11(7.9)	41(29.1)	52(37)	
	Yes	5 (3.6)	82(59.4)	87(63)	
	Total	**16(11.5)**	**123(88.5)**	**139(100)**	
aadA5	No	11(7.9)	49(35.2)	60(43.1)	0.028
	Yes	5(3.6)	74(53.3)	79(56.9)	
	Total	**16(11.5)**	**123(88.5)**	**139 (100)**	
aadA2	No	15(10.8)	113(81.3)	128(92.1)	0.000
	Yes	1(0.7)	10(7.2)	11(7.9)	
	Total	**16(11.5)**	**123(88.5)**	**139(100)**	

AGMG* = Aminoglycosides modifying enzymes genes, Fisher exact test (P-value)

Fluoroquinolones and quinolones: genetic factors of quinolone resistance protein were one of the least prevalent antimicrobial resistance genes identified, *QnrS1* (n=08), *qnrB* (n=01), *qnrB4* (n=01), and *qepA* (n=01). The *aac(6')-Ib-cr* that encode resistance for fluoroquinolones is most prevalent. Strains encoding *qnr* genes also encoded *bla*$_{\text{CTX-M-15}}$ genes and several genetic determinants of resistance to other classes of antimicrobials.

Sulfonamides: the *sul1* and *sul2* genes were commonly identified in most of the isolates and *sul3* was found only in one strain. Among resistance genes for sulfonamides *sul1*(68.3%) is most prevalent and followed by *sul2* (57.5%). Prevalence of sulfonamides genes, encoding both *sul1* and *sul2* is 50(35.9%), *sul1* (32.4%), *sul2* (21.6%), and 10% of the strains have not encoded any resistance genes for sulfonamides.

Trimethoprim: multiple variants of trimethoprim resistance genes were identified. These resistance genes include: *dfrA*, *dfrA12*, *dfrA17*, *dfrA27*, *dfrA5*, *dfrA7*, *dfrA8*, *dfrB4.* The most prevalent trimethoprim resistance gene, *dfrA17* was 81/139(58.3%) In 25/139 (17.9%) of these strains any variant of trimethoprim resistance genes was not detected.

Generally, higher prevalence of resistance genes were identified fo commonly prescribed antibiotics at the hospital either in combination or alone. Most of the strains MDR and resistant to three and morethan three classes of antimicrobials.

Table 3.10: Strains of most prevalent sequence types and associated resistance genes

ST (n)	*bla*$_{CTX-M-15}$ (%)	*bla*$_{TEM-1B}$ (%)	*bla*$_{OXA-1}$ (%)	*bla*$_{CMY-2}$ (%)	*bla*$_{CMY-42}$ (%)	*aac(6')-Ib-cr* (%)	*QnrS1* (%)	*dfrA17* (%)	*sul1/sul2* (%)	*catB3* (%)
ST410(21)	20(95.8%)	12(57.1%)	19(90.4%)	17(80.9%)	-	19(90.5%)	-	*18(85.7%)	17(80.9%)	18(85.7%)
ST648(15)	15(100%)	13(86.6%)	14(93.3%)	-		14(93.3%)	-	*15(100%)	15(100%)	13(86.6)
ST131(8)	8(100%)	4(50%)	7(87.5%)	-		6(75%)	-	4(50%)	8(100%)	4(50%)
ST10 (9)	8(88.9%)	7(77.8%)	2(22.2)	-		2(22.2%)	1	8(88.8%)	3(33.3%)	2(22.2%)
ST2659(8)	8(100%)	-	8(100%)	-		8(100%)	-	7(87.5%)	7(87.5%)	8(100%)
ST38 (6)	6(100%)	1(16.6%)	5(83.3%)	-		2(33.3%)	-	6(100%)	3(50%)	2(33.3%)
ST167(7)	6(85.6%)	3(42.8%)	4(57.1%)	-	1(14.2)	4(57.1%)	-	*4(57.1%)	7(100%)	3(42.8%)
ST405 (6)	5(83.3%)	1(16.6%)	6(100%)	-		5(83.3%)	-	5(83.3%)	1(16.6%)	4(66.6%)
ST130 (5)	5(100%)	5(100%)	-	-		-	-	5(100%)	5(100%)	-
ST44 (4)	4(100%)	1(25%)	4(100%)	-		4(100%)	-	4(100%)	4(100%)	4(100%)
ST2172 (3)	3(100%)	2(66.6%)	-	-		-	-	-	-	-
ST617 (4)	3(75%)	2(50%)	4(100%)	-		4(100%)	-	4(100%)	4(100%)	4(100%)
ST58 (3)	2(50%)	2(66.6%)	1(33.3)	-		1(33.3%)	1	2(66.6%)	2(66.6%)	1(33.3%)
ST2851(2)	2(100%)	2(100%)	1(50%)	-	1(50%)	1(50%)		1(50%)	1(50%)	1(50%)

Key: - ST: Sequence type, *sum of other variants too,

Prevalent sequence types of *E. coli* strains identified encoding multiple antimicrobial resistance genes. One of the least prevalent resistance genes, *bla*$_{CMY-2}$ was exclusively associated to most prevalent ST410, and CMY-42 another variant of CMY-2 was found only in two strains that belong to two different sequence types (ST2851 and ST167). These prevalent sequence types were multiple resistant strains. Moreover, strains with these sequence types were international clones reported from different sources including clinical and environmental samples from different parts of the world. Multiple antimicrobial resistance genes were identified from strains with the most frequent MLST types (**Table 3.10**).

3.2.5 Plasmids and replicon typing

Plasmid replicon typing was performed by using *in silico* whole plasmid genome detection and characterization tool called *PlasmidFinder1.3*, Center for Genomic Epidemiology database (https://cge.cbs.dtu.dk/services/PlasmidFinder/). Curated for characterizing/replicon typing of plasmids usually encoded by the order *Enterobacterales.*

More than fifteen different plasmid replicon types were identified, these include plasmids that are broad-host-range and capable of carrying and transfer MDR genes/functions, and those plasmids that have a capacity to carry and transfer both MDR and virulence genes/functions. Multiple copies of a single plasmid replicon types were encountered in a single strain, and multiple copies of different plasmid replicon types were also identified in many of these strains. The predominant replicon types were *IncFII* (pRSB107 32/97(33%), *IncQ1* 26/97(27%) and *Col* (BS512**)** 18/97**(**10%). The overall prevalence of plasmid replicons among all strains of *E. coli* was *IncFII (pRSB107* (24.8%), *IncQ1* (20%) and *Col* (BS512**) (**14%) (**Table 3.11**). Furthermore, the *IncFII*(pRSB107) replicons were identified from *E. coli* strains with seven different sequence types, ST648 (37.5%), ST130 (18.7%), and ST38 (15.6%).

IncQ1, the second most prevalent replicon type identified from at least eight sequence types. But these plasmids were predominantly identified from ST410 (61.5%, n=13) and ST2127 (11.5%, n=3). *Col*(BS512), third most prevalent replicon type was detected from nine different sequence types, and these replicons were relatively common among ST405 (22.2%), ST648 (22.2%), and ST2659 (16.7%). For general understanding of plasmids detected from strains in this study, details regarding the functional annotations of these plasmids were extracted from GenBank. Main classes/category of plasmids, functional annotations, and GenBank accession numbers, were summarized below (see **Table 3.12**).

Table:3.11: Distribution of plasmid replicon types in different STs of *E. coli* strains

S.no	Replicon types	Freq. (%)	STs (n)
1.	*IncFII(pRSB107)*	32(24.8%)	ST648(12), ST130(6), ST38(5), ST131(3), ST2127(3), ST5017(1) ST Novel (2)
2.	*IncQ1*	26(20%)	ST410(16), ST2127(3), ST448(1), ST394(1), ST90(1), ST44(1), ST515(1), Novel (2)
3.	*Col(BS512)*	18(14%)	ST405(4), ST648(4), ST2659(3), ST410(2), ST44(1), ST130(1), ST167(1), ST5017(1), ST394(1)
4.	*IncX4*	10(7.8%)	ST405(3), ST2659(2), ST648(1), ST410(1), ST167(1), ST5342(1), ST2178(1)
5.	*IncI1*	8(6.2%)	ST44(1), ST205(1), ST394(1), ST10(1), ST617(1), ST58(1), ST167(1) Novel (1)
6.	*IncFII*	7(5.4%)	ST648(1), ST405(1), ST46(1), ST10(1), ST58(1), Novel (2)
7.	*IncA/C2*	5(3.9%)	ST69(1), ST58(1), ST90(1), ST167(1), Novel(1)
8.	*IncHI2A*	5(3.9%)	ST2851(2), ST1434(1), ST409(1), ST410(1)
9.	*IncI2*	5(3.9%)	ST648(2), ST90(1), ST10(1), ST1423(1)
10.	*IncHI2*	4(3.1%)	ST410(1), ST409(1), ST1434(1), ST2851(1)
11.	*IncB/O/K/Z*	2(1.6%)	ST1284(1), ST58(1)
12.	*IncR*	2(1.6%)	ST205(1)
13.	*IncFIB (AP001918)*	2(1.6%)	ST44(1), ST10(1)
14.	*IncFIA*	2(1.6%)	ST58(1), ST131(1)
15.	*IncFIB(pQil)*	1(0.8%)	Novel (1)
Grand total		**129(100%)**	

ST: Sequence type, (n): number of strains in that ST that the plasmid

Table-3:11-shows overall prevalence of plasmid replicon types identified and the distribution of the replicon type among the predominant sequence types. It also shows that the distribution of certain *Inc*-type across a number of different sequence types. Most of the plasmids are broad-host-range by their nature. The *IncQ1* replicon plasmids were most frequently identified from ST410 than others sequence types, however, It was has been also detected from other sequence types as well.

Table 3.12: **Annotations of plasmid replicons in *Enterobacterales* as extracted from GenBank**

S.no	Plasmid (rep)	Description/functional	GenBank accession no.
1.	*Col (BS512)*	Colicins	http://www.ncbi.nlm.nih.gov/nuccore/NC_010656
2.	*Col156*	Colicins	http://www.ncbi.nlm.nih.gov/nuccore/NC_009781
3.	*Col8282*	Colicins	http://www.ncbi.nlm.nih.gov/nuccore/DQ995353
4.	*IncA/C2*	Broad host range plasmids capable of carrying transfer and MDR functions	http://www.ncbi.nlm.nih.gov/nuccore/JN157804
5.	*IncB/O/K/Z*	Plasmids capable of carrying and transfer, MDR and virulence functions	http://www.ncbi.nlm.nih.gov/nuccore/FN868832
6.	*IncFIA*	Plasmids capable of carrying and transfer MDR, and virulence functions	http://www.ncbi.nlm.nih.gov/nuccore/AP001918
7.	*IncFIB*	Plasmids capable of carrying and transfer, MDR and virulence functions	http://www.ncbi.nlm.nih.gov/nuccore/AP001918
8.	*IncFIB (PB171)*	Plasmids capable of carrying and transfer, MDR and virulence functions	http://www.ncbi.nlm.nih.gov/nuccore/AB024946
9.	*IncFIB(pQil)*	Plasmids capable of carrying and transfer, MDR and virulence functions	http://www.ncbi.nlm.nih.gov/nuccore/JN233705
10	*IncFII*	Plasmids Capable of carrying resistance genes and virulence determinants	http://www.ncbi.nlm.nih.gov/nuccore/AY458016
11	*IncFII (29)*	Plasmids Capable of carrying resistance genes and virulence determinants	http://www.ncbi.nlm.nih.gov/nuccore/CP003035
12	*IncFII(K)*	Plasmids capable of carrying and transfer MDR functions	http://www.ncbi.nlm.nih.gov/nuccore/CP000648
13	*IncFII(pRSB107)*	Plasmids Capable of carrying resistance genes and virulence determinants	http://www.ncbi.nlm.nih.gov/nuccore/AJ851089
14	*IncHI2*	Plasmids capable of carrying and transfer MDR functions	http://www.ncbi.nlm.nih.gov/nuccore/BX664015
15	*IncHI2A*	Plasmids capable of carrying and transfer MDR functions	http://www.ncbi.nlm.nih.gov/nuccore/BX664015
16	*IncI1*	Plasmids capable of carrying and transfer MDR functions	http://www.ncbi.nlm.nih.gov/nuccore/AP005147
17	*IncI2*	Plasmids capable of carrying and transfer MDR functions	http://www.ncbi.nlm.nih.gov/nuccore/KP347127
18	*IncQ1*	Broad host range plasmids capable of carrying MDR functions	http://www.ncbi.nlm.nih.gov/nuccore/HE654726

19	*IncR*	Broad host range plasmids capable of carrying MDR functions	http://www.ncbi.nlm.nih.gov/nuccore/DQ449578
20	*IncX1*	Plasmids capable of carrying and transfer MDR functions	http://www.ncbi.nlm.nih.gov/nuccore/EU370913
21	*IncX3*	Plasmids capable of carrying transfer and MDR functions	http://www.ncbi.nlm.nih.gov/nuccore/JN247852
22	*IncX4*	Plasmids capable of carrying and transfer MDR functions	http://www.ncbi.nlm.nih.gov/nuccore/CP002895
23	*IncY*	Phage like plasmids	http://www.ncbi.nlm.nih.gov/nuccore/K02380
24	*p0111*	EHEC virulence plasmid	http://www.ncbi.nlm.nih.gov/nuccore/AP010962

3.2.6 Virulence genes and pathotypes of ESBL-producing *E. coli* strains

Putative virulence genes were extracted by using virulence gene finder database (web-tool) called *VirulenceFinder ver.1.5.* at (*https://cge.cbs.dtu.dk/services/VirulenceFinder/)*. The virulence factors identified among these strains were grouped in to five categories as adhesins, toxins, protectins, siderophores, invasins, and another miscellaneous group. Acquisition of some specified virulence genes can be related to a particular pathotype. According to some literatures, there are unique genetic determinants of virulence that are associated to certain pathotypes of *E. coli*. Genetic determinants of virulence factors identified in these *E. coli* strains and their putative pathotypes are summarized (**Table 3.14**).

Based on Clement phylotype analysis, these strains were classified into several phylogroups (A (28%, 40/127), B1 (8.6%, 12/127), B2 (6.4%, 10/127), C (17.9%, 22/127), D (17.2%, 23/127), E (5.0%, 6/127) and F (10.8%, 14/127)). A set of virulence gene that can be used to group the strains into different pathotypes were detected. These putative function of these virulence genes were identified as: adhesins, protectins, toxins, siderophores, invasins and other miscellaneous virulence genes. Pathotyping was determined by the presence of a set of virulence genes that defines a particular pathotype. These set of virulence genes has been described in a previous publication (Croxen *et al*., 2013). Accordingly these strains are classified in to five pathotypes. EPEC (46%, 64/139), ExPEC/UPEC (33.1%, 46/139), EAEC (12.9%, 18/139), ETEC (2.9%, 5/139), and other miscellaneous DEC (5.1%, 7/139).

Generally, robust virulence genes were detected in most of the strains based on analysis of virulence genes on virulence factor database for pathogenic bacteria (VFDB). However, toxin and/or toxin-like virulence genes were less prevalent in ST410 group of strains. A statistical analysis showed that toxin and/or toxin-like virulence genes were not associated to ST410 as compared to non–ST410 (p-value = 0.0002). Though there are other strains/sequence types isolated from the pediatric population carrying toxin/toxin-like virulence genes, ST410, the most prevalent sequence type among isolates from pediatric unit were not associated to toxins. These strains also encode pathotype defining virulence genes and most of these strains were determined as EAEC (**Table 3.13**). However, none of the strains in this study has encoded shiga-toxin/verotoxin.

Table 3.13: ST410 and virulence genes among *E. coli* collections

	Presence or absence of toxin or toxin-like virulence factors			
Sequence type (ST)	Yes	No	Total	*P-value
ST410	3	18	21	0.0002
Non-ST410	69	49	118	
Total	72	67	139	

Fisher exact test (p-value= 0.0002),

Table 3.14: Virulence genes identified and most related *E. coli* pathotypes

Category	Comment		Location	Pathotypes	Ref.
Adhesins/ Adherence	*aagA*	Fimbrial Subunit encoding AAF/I	Plasmid	DEC (EAEC)	(Croxen et al., 2013)
	iha	Adhesin siderophores	Chromosome	ExPEC, STEC	(Johnson and Russo, 2005)
	perA	EPEC adherence factor	Plasmid	EPEC	(Contreras et al., 2010)
	lpf	Long polar fimbriae	Chromosome	ExPEC, DEC (EHEC, EPEC, EAEC)	(Hu et al., 2015)
Toxins	*astA*	Enteroaggregative heat labile enterotoxin EASI1	Chromosome/ plasmid	ExPEC, DEC (EAEC, EPEC, ETEC)	(Johnson and Nolan, 2009)
	sat	Secreted auto transporter toxin	Chromosome/ plasmid	ExPEC, DEC (EAEC, EPEC, ETEC)	(Johnson and Russo, 2005)
	pic	Serine protease autotransporters of *Enterobacteriaceae* (SPATE)	Plasmid	ExPEC, DEC (EAEC, EPEC, ETEC)	(Abreu et al., 2015)
	senB	Plasmid encoded enterotoxin	Plasmid	UPEC	(Cusumano et al., 2010)
	cma	Colicin M	Plasmid	ExPEC, DEC	(Cascales et al., 2007)
	cnf1	Cytotoxic necrotizing factor	Chromosome	ExPEC	(Johnson and Russo, 2005)
Protectins	*iss*	Increased serum survival	Chrom. and Plasmid	ExPEC	(Johnson and Nolan, 2009)
	mchc	MchC protein	Chromosome/ plasmid	EHEC, EPEC	(Gonzalez-Escalona et al., 2016; Poey et al., 2006)
	mchf	ABC transporter protein MchF	Chromosome/ plasmid	EHEC, EPEC	(Gonzalez-Escalona et al., 2016; Poey et al., 2006)
	mcmA	Microcin M part of colicin H	Chromosome/ plasmid	ExPEC, EHEC,	(Gonzalez-Escalona et al., 2016; Poey et al., 2006)
Siderophores	*capU*	Protein resembling O157:H7 LPS	Plasmid	DEC (EAEC)	(Lima et al., 2013)
	ironN	Siderophore receptor	Plasmid	EPEC	(Johnson and Russo, 2005)
	ireA	Siderophore receptor	Chromosome	ExPEC	(Johnson and Russo, 2005)
Invasins	*aap*	Dispersin/ anti-aggregation protein	Plasmid	ExPEC DEC(EAEC)	(Boisen et al., 2012)

Others	*aaiC*	aggR-activated island encoding a type VI secretion system	Chromosome	DEC (EAEC)	(Boisen et al., 2012)
	aar	aggR-activated regulator, AraC-negative regulator	Plasmid	DEC (EAEC, ETEC)	(Santiago et al., 2014)
	aggR	Master regulator	Plasmid	DEC (EAEC)	(Boisen et al., 2012; Johnson and Nolan, 2009)
	gad	Glutamate decarboxylase	Chromosome	ExPEC, EHEC, DEC	(Grant et al., 2001)
	agg3D	Chaperone, AAF/III assembly unit	Plasmid	DEC (EAEC)	(Jønsson et al., 2015)
	eilA	Salmonella HilA homolog	Chromosome	EAEC	(Sheikh et al., 2006)

Key: ExPEC: Extra intestinal Pathogenic *E. coli*, EPEC: Enteropathogenic *E. coli*, EAEC: Enteroaggregative *E. coli*, EHEC: Enterohemorrhagic *E. coli*, ETEC: Enterotoxigenic *E. coli*, UPEC: Uropathogenic *E. coli*, STEC: Shigatoxin producing *E. coli,* DEC: diarrheagenic *E. coli.*

3.2.7 Serotypes/Serogroups of *E. coli* strains

Phenotypic serotyping or typing using antisera is one of the techniques previously used for epidemiological typing, diagnosis, treatment, and prevention of bacterial strains. But now, once whole genome data is available there are multiple different options are apparent for typing of a bacterial strain. Molecular serotyping more specifically serotype prediction is performed by comparing the draft genome of the test strain to the of genome of the reference strains in the database. *In silico*, bioinformatics tool for *E. coli* serotype prediction, ***serotypeFinder*1.1** (https://cge.cbs.dtu.dk/services/SerotypeFinder/) from Center for Genomic Epidemiology was used.

A total of over 50 serotypes were determined including non-typeable strains. Some of them were previously reported as pathogenic strains that included the very known ExPEC serotype O25:H4. Serogroups O8, 89, O25, O2, O102, O176, O9 and O15 were the most common serotypes among the strains (**Figure 3-3**).

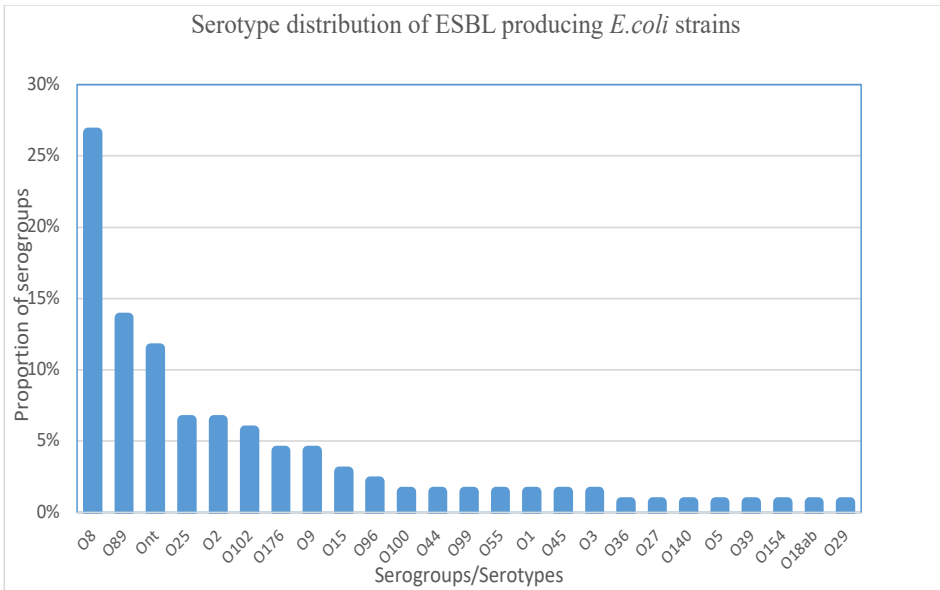

Figure 3.3: Distribution of predicted serotypes among *E. coli* collections at JMC, 2016.

3.2.8 Multi-Locus Sequence Typing (MLST)

Multi-locus-sequence typing (MLST) is most important and widely used microbial typing method, often considered golden standard for strain typing. MLST was performed by using a web-tool that uses a draft whole genome: *MLST ver 2.0* nested in Center for Genomic Epidemiology database and hosted at the Technical University of Denmark (DTU).

An assembled draft genome uploaded to the database and sequence types were retrieved. More than thirty different sequence types were identified among *E. coli* collection. The most frequent MLST/STs include: ST410 (23%, 21/86), ST648 (17%, 15/86), ST 131(10%, 9/86), ST10 (9%, 8/86), ST2659 (9%, 8/86), ST38 (7%, 6/86), ST167 (7%, 6/86), both ST130 and ST405 each account for **(**6%, 5/86) of the strains, and ST44 (5%, 4/86)) (**Figure 3-4**). A few other strains not shown in this figure were either single strain represents the ST, or novel ST.

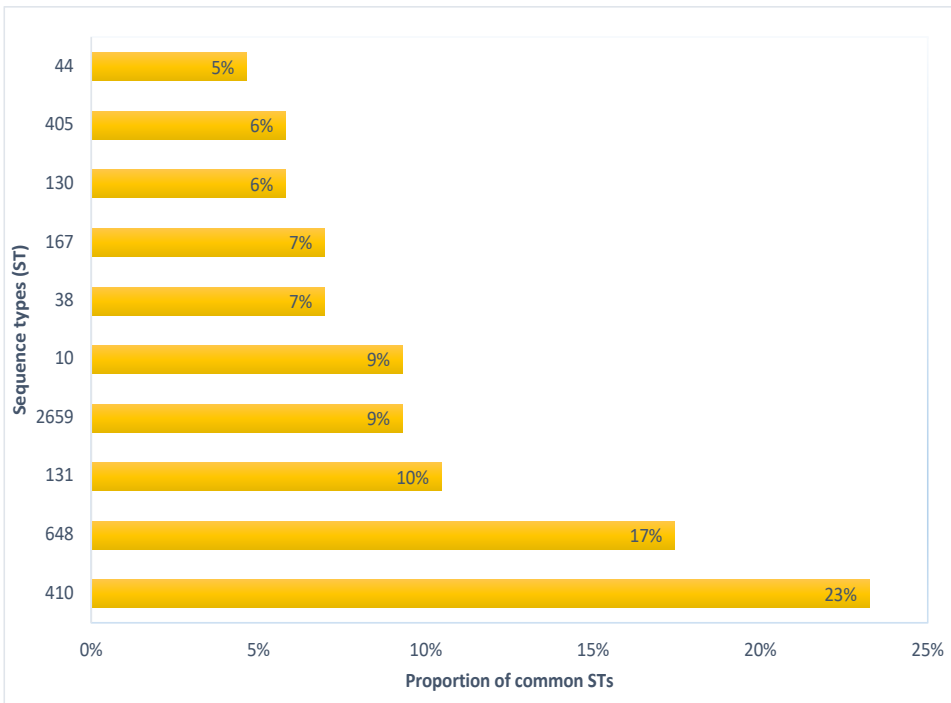

Figure 3.4: **Prevalent MLSTs among *E. coli* strains collected at JMC, 2016**

Table 3.15: Strains with multiple virulence, MLSTs, serotypes and putative pathotypes

#	Strain ID	ST	Serotype	Virulence genes	Putative pathotypes
1	**m020e**	131	O25:H4	*cma, iss, iroN, iha, sat, gad*	ExPEC
2	**m301e**	167	O89:H9	*cap, senB, iss*	ETEC
3	**p015e**	Novel	O25:H4	*agg3C, aggR, aap, aar, aggA*	EAEC
4	**p034e**	38	O15:H30	*aap, aggR, iss, aggA, aar*	EAEC
5	**p035e**	38	O99:H30	*agg3C, aggR, aap, aar, iss*	EAEC
6	**p042e**	38	O15:H30	*aap, iss, aggR, aggA, aar*	EAEC
7	p047e	38	O15:H30	*aap, aggR, iss, aggA, aar*	EAEC
8	**p060e**	2178	O39:H49	*pic, lpfA, astA*	ExPEC
9	**p066e**	38	O3:H30	*aap, aggR, agg3C, iss, aar*	EAEC
10	**p070e**	38	O3:H30	*aap, aggR, agg3C, iss, aar*	EAEC
11	**p073e**	131	O25:H4	*iss, sat, ireA, iha, SenB*	ExPEC
12	**p098e**	130	O176:H34	*mchC, aggR, mchF, mcmA, pic, astA, aaiC, aap, agg3D, lpfA*	EAEC
13	**p100e**	130	O176:H34	*aggR, lpfA, aaiC, astA, mcmA, mchF, aap, pic, agg3D, mchC*	EAEC
14	**p103e**	130	O176:H34	*aap, astA, mcmA, aggR, aaiC, mchF, lpfA, agg3D, mchC, pic*	EAEC
15	**p105e**	130	O176:H34	*mchC, aggR, mchF, mcmA, pic, astA, aaiC, aap, agg3D, lpfA*	EAEC
16	**p116e**	58	O8:H30	*capU, lpfA, senB,*	ETEC
17	**p120e**	130	O176:H34	*aap, mchC, mchF, mcmA, pic, aggR, aaiC, lpfA, agg3D, astA*	EAEC
18	**p154e**	69	O15:H18	*aar, lpfA, eilA, iha, iss, aggR, aap*	EAEC
19	**s097e**	131	O25:H4	*cnf1, sat, iss, SenB, iha*	ExPEC
20	**s101e**	131	O25:H4	*senB, sat, iha,*	ExPEC
21	**s106e**	Novel	O45:H16	*lpfA, iha, eilA, astA, iss*	ExPEC
22	**s140e**	131	O25:H4	*cnf1, iss, iha, senB, sat*	ExPEC
23	**s167e**	58	O8:H25	*iroN, iss, lpfA,*	ExPEC
24	**s177e**	131	O25:H4	*cnf1, iss, iha, sat*	ExPEC
25	**s184e**	131	O25:H4	*cnf1, iss,iha, sat, senB*	ExPEC
26	**s351e**	131	O25:H4	*cma, iha, sat*	ExPEC

Key: The above table shows commonest STs with toxin/like virulence genes and putative pathotypes for strains from different units (Medical:mXXXe, Pediatric:pXXXe, Surgical: sXXXe. In this table, multiple virulence genes were mainly associated to strains from pediatric unit and EAEC pathotypes. Virulence genes related to EPEC and ExPEC pathotypes were identified: Extracted from VirulenceFinder at CGE: **https://cge.cbs.dtu.dk/services/VirulenceFinder/**

3.2.9 Epidemiological lineages and population genetics

Predominant sequence types were ST410 (23%), ST648(17%), ST10(9%) and ST2659 (9%), ST131(8%), and ST167(8%). The prevalent clonal complexes ST10-complex 24(18.9%), ST23-complex 19(14.9%), ST648-complex 12(9.4%), and ST131-complex 7(5.5%). These complexes are international clones reported from several other countries, and epidemiological strains related to epidemiologic factors (**Figure 3-5**).

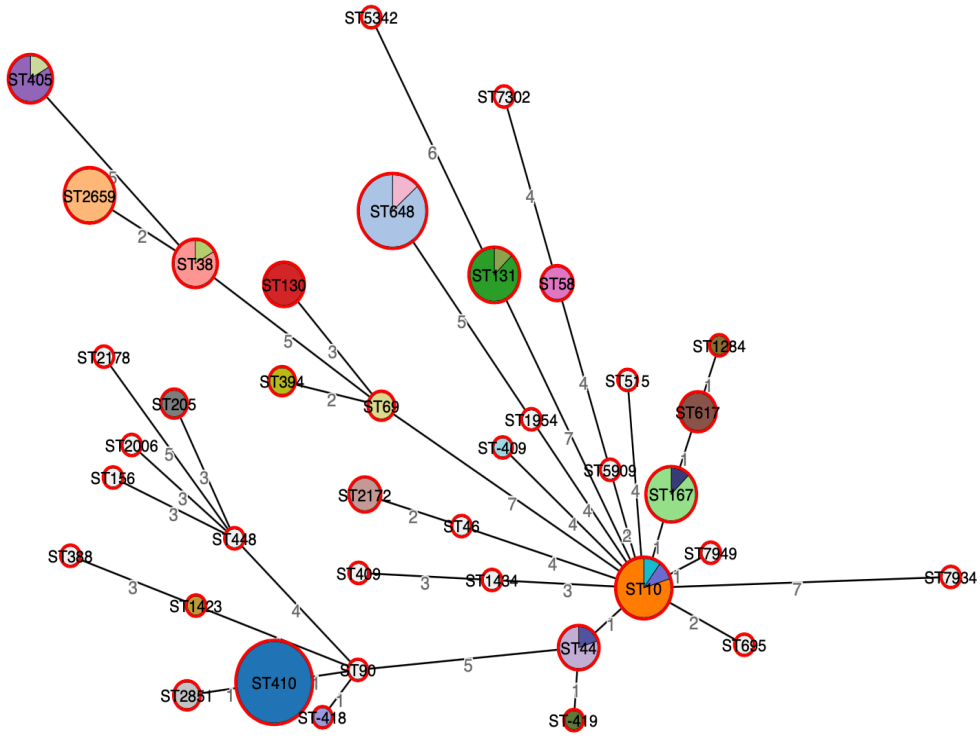

Figure 3.5: Minimum spanning tree of *E. coli* strains

A Minimum Spanning tree (MS-treeV2) analysis demonstrated that some sequence types clustered together in a classical seven gene MLST. Overall there were over 30 sequence types identified, some of them were in clusters and many strains also existed as singleton. ST410 is the most frequently isolated sequence type.

The pandemic lineage -ST131-H30-Rx

An epidemiological *E. coli* strain, O25:H4-ST131-$H_{30\text{-}Rx}$ was identified in our collections. Five out of seven (71.4%) of O25:H4-ST131-$H_{30\text{-}RX}$ strains were isolated from surgical ward, two from surgical site infection and two from other types of wound infection, one from a patient diagnosed with urinary tract infection who had developed urethral stricture in the hospital. The other two strains were isolated from patients at medical ward and pediatric ward. All specimens were collected from admitted patients.

cgMLST based phylogenetic tree, revealed clustering of ST131-H30-Rx Ethiopian strains with ST131-H30-Rx from Europe (**Figure 3.6**).

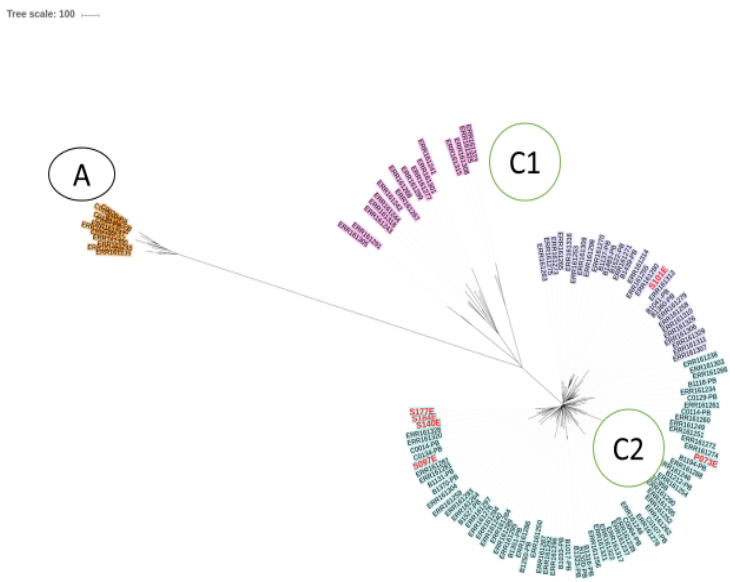

Figure 3.6: A cgMLST based cluster of ST131-H30-Rx *E. coli* strains:

A cgMLST tree showing ST131-H30-Rx strains from Ethiopia (Red) clustering with ST131-H30-Rx strains collected from Europe. In this tree, cluster (A) belong to the A sub-clone of ST131, and C1 and C2 are clades that belong to the C sub-clone. The ST131-H30-Rx belongs to the C2 clades as indicated above in the figure. ST131-C2 sub-clone usually characterized by the presence of *pap* genes, *afa/dra* adhesins, *kspMII* and *iutA*aerobcatin genes. Also based on other observed genes, and according to a study by Blanco *et al*, 2013; these strains were classified into two virotypes: one strain belong to Virotype **B** (*cma, iss, iroN, iha, and sat genes*) and all the rest belong to virotype **C** (*senB*, *iss*, *ireA*, *cnf1, iha*, and *sat*).

On the other hand, SNP based phylogenetic analysis revealed a likely outbreak of the *bla*$_{CTX\text{-}M\text{-}15}$-producing strains. Some strains clustered together with very few (often from 0 to less than 10 SNPs) (**Figure 3.7**).

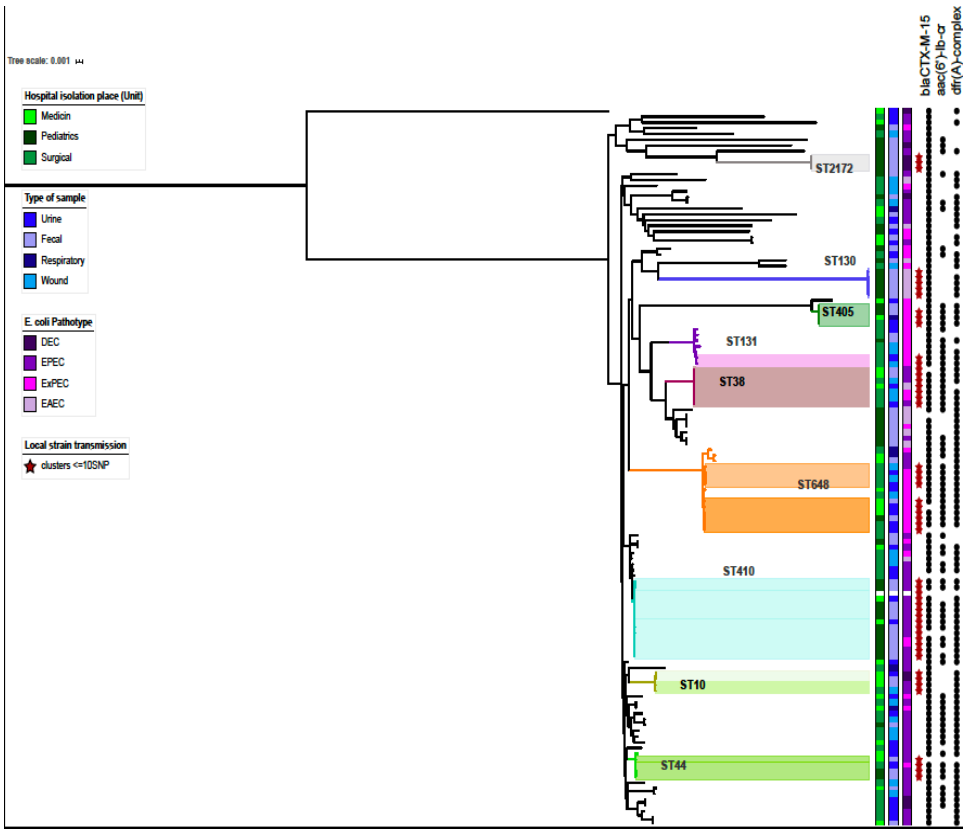

Figure 3.7: Clusters of international clones at different units in the hospital

A SNP-based phylogenetic analysis of the dissemination of strains in hospital wards revealed that several clusters of international clones that differ a maximum of less than 10 SNPs. These strains were isolated from different patient categories, disease conditions and wards. The clusters include international clones like ST131, ST410, ST648, ST130, ST44, ST10, ST2172, ST38 and ST405. Though most of these strains were clustered into an international clone, there were also many numbers of singletons.

The tree revealed that the dissemination of the resistance genes was along several lines of clones, and in all the units of the hospital. Furthermore, these strains (both clusters and non-clustered strains) were associated with presence of several antimicrobial genes (**Figure 3-8**).

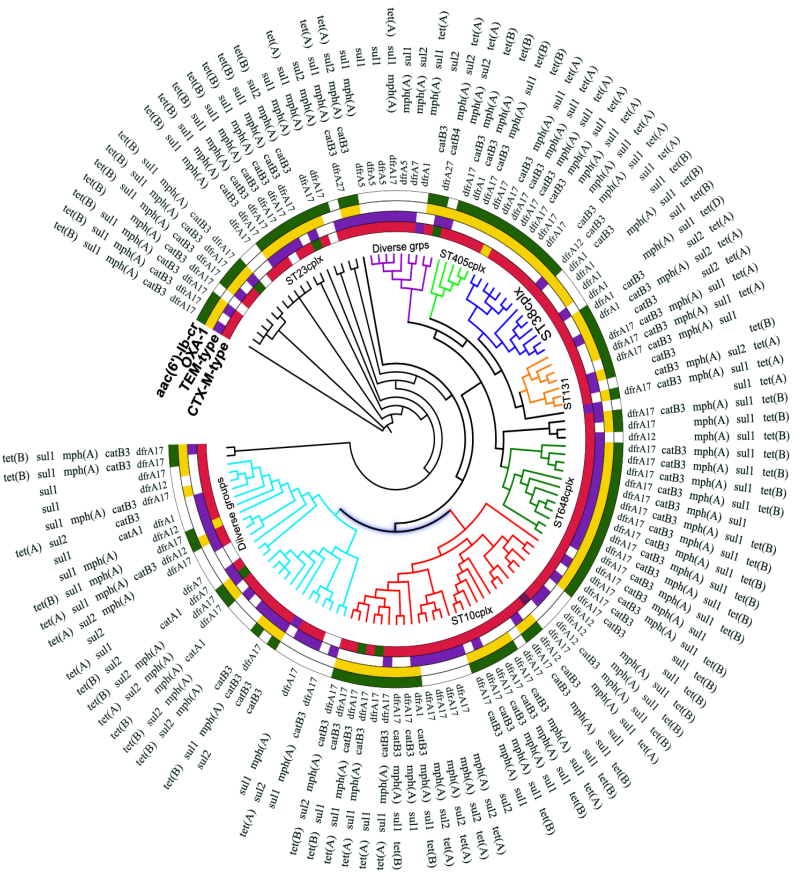

Figure 3.8: A phylogenetic tree of *E. coli* strains encoding for multiple resistance genes

In a phylogenetic tree generated based on SNP (**Figure 3-8**), all colored clades show clustered strains in general, and the different colors show different clusters/sequence types (STs). The inner strip (mostly red): *bla*$_{\text{CTX-M}}$-types, second- inner (purple): *bla*$_{\text{TEM-1B}}$): third- inner strip (yellow): *bla*$_{\text{OXA-1,}}$ and the fourth-inner strip: *aac(6')-IB-cr* genes. And all other antimicrobial genes were represented by the genes identified. These genes include (*sul1* and *sul2*: sulfonamides, *dfrA17*: trimethoprim, *mphA*: macrolides, *catB3*: phenicol's, and *tetA* and *tetB*: tetracyclines).

3.3 Phenotypic and molecular features of *K. pneumoniae* strains

3.3.1 Phenotypic features of *K. pneumoniae* strains

A total of 146 *K. pneumoniae* strains collected from different clinical specimens, sputum, urine, wound swab, and diarrheic stool. These specimens were collected from patients either admitted to the hospital or visiting as outpatient service at medical unit, surgical unit, pediatric unit, and intensive care unit (ICU)). Seventy six percent (n=111) of these strains were ESBL-producing, and 22.6%(n=33) of them were phenotypically non-susceptible to carbapenems (**Table 3-16**). The rate of resistance to non-β-lactam classes of antimicrobials were also very high. And hence, most of the isolates were multiple drug resistant strains, all strains were resistant to at least two class of antimicrobials.

Table 3.16: Rate of ESBL and carbapenem non-susceptible *K. pneumoniae* at JMC

ESBL phenotype	**Frequency**
Yes	**111(76%)**
No	35(24%)
Total	**146(100%)**
Resistance to carbapenems	**Frequency**
Yes	**33(22.6%)**
No	113(77.4%)
Total	**146(100%)**

All isolates were tested for reduced susceptibility to carbapenem antimicrobials. Though, 22.6% were carbapenem non-susceptible, only one strain was a carbapenemase producer. The ESBL strains carry also multiple resistance genes for both β-lactam antimicrobials and non-β-latam antimicrobials. The genetic determinants of non-β-lactam antimicrobilas is from both within, and between different classes of antimicrobials. Based on a phenotypic study, most of these *Klebsiella* strains were resistant to aminoglycosides (81.8% (n=90) for gentamicin, 4.5% (n=5) for amikacin), ciprofloxacin (62.7%, n=70), sulfamethoxazole (94.5%, n=105), and piperacillin-tazobactam (50%, n=56). All the isolates were resistant to at least two classes of antimicrobials and most of these strains (75%) were resistant to at least four classes of antimicrobials (**Figure 3.9**)

3.3.2 Antibiogram and rate of MDR

All strains of *bla*$_{CTX-M-15}$-producing *K. pneumoniae* strains were multidrug-resistant. More than 93%(n=104) of strains were resistant to at least three classes of antimicrobial including β-lactams and non-β-lactams.

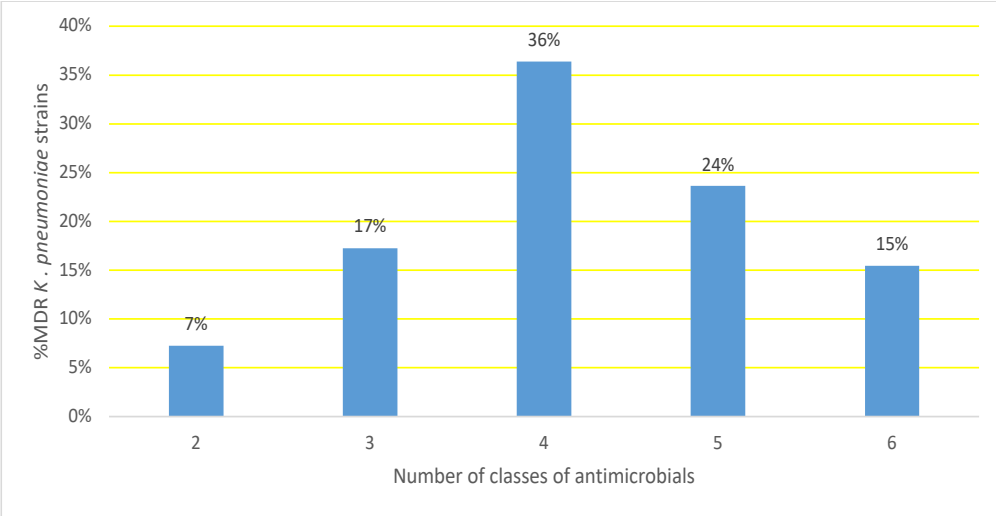

Figure 3.9: Rate of MDR among *K. pneumoniae* strains from clinical specimens at JMC

The phenotypic antibiogram data **(Table 3.17** shows the proportion of resistant strains for certain number antibiotics in a given class of antimicrobials and mainly the number of classes of antimicrobials.

From this data, it can be shown that several of these strains are MDR, resistant to different classes of antimicrobials. The starins showed lower rates of resistance to carbapenems. Among carbapenem non-susceptible strains, only one strain encoded the metallo-β-lactamase (*bla*$_{NDM-1}$), as genetic determinant of carbapenemases. Overall, the least rate of resistance was observed for carbapenems.

Table 3.17: Antibiogram of ESBL-producing *K. pneumoniae* at JMC

Strains (%)	Antibiogram of ESBL strains of *K. pneumoniae* (%)	#of drugs	# classes of antimicrobials
08(7.2%)	***CTX-CAZ-SXT (62.5%)***	3	2
	CTX-CAZ-CN (12.5%)		
	CTX-CAZ-CN (25%)		
19(17.2%)	***CTX-CAZ-SXT-CN (57.9%)***	4	3
	CTX-CAZ-CIP-SXT (26.3%)		
	CTX-CAZ-MEM-CN (5.2%)		
	CTX-CAZ-SXT-MEM-CN (10.5%)	5	
40(36.3%)	*CTX-CIP-SXT-CN (2.5%)*	4	4
	CTX-CAZ-CIP-SXT-CN (35%)	5	
	CTX-CAZ-PTZ-SXT-CN (32.5%)		
	CTX-CAZ-CIP-SXT-MEM (12.5%)		
	CTX-CAZ-SXT-MEM-CN (10%)		
	CTX-CAZ-PTZ-CIP-SXT (5%)		
	CTX-CAZ-PTZ-CIP-AK (2.5%)		
26(23.6%)	*CTX-PTZ-CIP-SXT-CN (3.8%)*	5	5
	CTX-CAZ-PTZ-CIP-SXT-CN (73%)	6	
	CTX-CAZ-CIP-SXT-MEM-CN (19.2%)		
	CTX-CAZ-PTZ-SXT-MEM-CN (3.8%)		
17(15.5%)	***CTX-CAZ-PTZ-CIP-SXT-MEM-CN (94.2%)***	7	6
	CTX-CAZ-PTZ-CIP-SXT-ETP-MEM-CN-AK (5.8%)	9	

CTX: Cefotaxime, PTZ: Piperacillin-Tazobactam, CIP: Ciprofloxacin, CAZ: Ceftazidime, AK: Amikacin SXT: sulfamethoxazole, ETP: Ertapenem, MEM: Meropenem, IPM: Imipenem, CN: Gentamycin

K. pneumoniae strains were tested against different types of drugs both from the same class and different classes. The above table shows that all strains were MDR strains, and (i) the first-column (% strains)- shows overall proportion of *K. pneumoniae* strains in that category (number of class of antimicrobials that strains were resistant to), (ii) second-column (antibiogram of ESBL strains) shows type of each antibiotic and specific drug in the combination and relative proportion in that combination with in that category of number of classes, (iii) third-column (#drugs)- shows number of drugs in combination that these strains were resistant to regardless of whether the antimicrobial agents are from the same class or not, (iv) fourth-column (#classes of antimicrobials)- shows a number of classes of antimicrobials that these *K. pneumoniae* strains were resistant to.

3.3.3 Molecular characteristics of *K. pneumoniae* strains

Molecular characteristics of ESBL-producing *K. pneumoniae* strains were analyzed after phenotypic identification of strains, first by conventional biochemical tests at JMC and later by MALDI-TOF mass spectrometer at Karolinska University Hospital. The *K. pneumoniae* strains were further confirmed for ESBL phenotype by disk diffusion tests. All strains with confirmed ESBL phenotype were selected for sequencing (for WGS on Illumina(Hiseq 2500) next generation sequencing platform). When the whole genome sequence was obtained strains were analyzed mainly with web-based genome analysis pipelines (databases) for analysis of *K. pneumoniae*.

Analysis of molecular features is focused mainly on molecular features of resistance genes (ESBL, carbapenemases, and also resistance genes for other classes of antimicrobials too), virulence genes (capsular types, lipopolysaccharides, siderophores, allantoin metabolism, protectins, and adhesins), MLST, and SNP-based phylogenetics of the bacterial population.

Resistance genes: resistance genes identified among these selected strains were primarily ESBL genes. However, other non-ESBL β-lactamases, and non-β-lactamase resistance genes like aminoglycosides, sulfonamides, fluoroquinolones, quinolones, trimethoprim, tetracycline, phenicol's, and macrolides were also detected.

Multiple resistance genes and their sub-variants were identified, the variants of ESBL, and other β-lactamases include: *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{SHV}}$. Generally, genetic determinants of carbapenem resistansce were less prevalent. Details of the prevalence of genes identified and their sub-variants are summaribzed in **Table 5-18** .

CTX-M-types: *bla*$_{\text{CTX-M}}$ was the most prevalent ESBL-type and *bla*$_{\text{CTX-M-15}}$ (84%, 92/109), followed by *bla*$_{\text{CTX-M-11}}$(3.6%, 4/109), *bla*$_{\text{CTX-M-14}}$ (1.8%), and (*bla*$_{\text{CTX-M-3}}$ and *bla*$_{\text{CTX-M-9}}$ each account for 1%). Only one strain carries two *bla*$_{\text{CTX-M}}$ genes (*bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15}}$). Whereas, 18.5% the strains were non-CTX-M types.

TEM-types: *bla*$_{\text{TEM}}$ genes were second most prevalent ESBL-genes, *bla*$_{\text{TEM-1b}}$ (69.7%, 76/109) was most prevalent, and *bla*$_{\text{TEM-1A}}$ (1.8) sub-variants were also detected, and TEM type variants were detected in 71.5%(78/109) of these strains.

SHV-types: multiple variants of *bla*$_{SHV}$-genes were detected, but these genes were relatively less prevalent as compared to other forms of ESBL-genes. The *bla*$_{SHV-11}$(21.1%, 23/109) was the most prevalent of the group followed by *bla*$_{SHV-1}$ (12.8%, 14/109), *bla*$_{SHV-28}$(6.3%, 7/109), *bla*$_{SHV-27}$ (3.6%, 4/109), and *bla*$_{SHV-26}$ (2.7%, 3/109).

OXA-types: another β-lactamase gene, *bla*$_{OXA}$-types were identified in 45%(50/109) of strains and these are *bla*$_{OXA-1}$(34.8%, 38/109), and *bla*$_{OXA-10}$(11%, 12/109).

CMY-types: *bla*$_{CMY}$-types were less prevalent detected, in only three strains, *bla*$_{CMY}$-6 (n=1, 0.9%) and CMY-42 (n=2, 1.8%) were the variants detected.

Carbapenemase: in the phenotypic analysis, 22.6%(n=33) were non-susceptible to carbapenem, but only 0.9% (n=1) of these strains encoded carbapenemases gene (the metallo-β-lactamase variant of carbapenemase, *bla*$_{NDM-1}$). Carbapenemase mediated resistance to carbapenems was the lowest rate of resistance detected.

Aminoglycosides: several variants of genes responsible for aminoglycosides resistance were identified. Most of the resistance genes for aminoglycosides detected were aminoglycoside modifying enzymes. The different variants of genes detected for aminoglycosides modifying enzymes includes those for acetylation, adenylation, phosphorylation, and streptomycin. The distribution of these genes and sub-variants of specific group vary in prevalence, and two or more of these enzymes co-existed within a genome of a single isolate. The types and prevalence of genetic determinants of aminoglycoside resistance were described in **Table 3.18**.

Aminoglycosides acetylation genes: several variants of the acetylase enzyme genes were detected and these genes were the most prevalent non-ESBL resistance genes. Most prevalent variants were *aac(6')-Ib-cr* (65.1%, 71/109), *aac(3)-IIa* (16.5%, 18/109), *aac(6')-IIc* (1.8%, 2/109), *aacA4*(1.8%, 2/109), *aac(3)-IId* (0.9%, 1/109).

Aminoglycosides adenylation genes: adenylation genes also appeared in four different variants that include*: aadA1*, *aadA2*, *aadA5*, and *aadE*. The prevalent variant of the group was *aadA2* (12.8%, 14/109). Adenylation genes were less frequent and more diverse in these *K. pneumoniae* strains.

Aminoglycosides phosphorylation genes: only one variant of phosphorylating enzymes gene was identified, *aph(3)-Ia* (9.2%, 10/109).

Aminoglycosides *strA* and *strB* genes: streptomycin resistance genes were identified in a half of strains. Both *strA* and *strB* genes account for 41.2% (45/109) each. It was very common to find both genes in the same strain.

Trimethoprim: acquired genes responsible for resistance of trimethoprim antimicrobials were detected in 61.3% of strains. Six variants of dihydrofolate reductase genes were identified. The *dfrA27* (32.1%) were most prevalent and followed by *dfrA7* (12.8%, 14/109), *dfrA12* (9.2%, 10/109), *dfrA1* (4.5%, 5/109), *dfrA17* (1.8%, 2/109)), and *dfrA5* (0.9%, 1/109).

Phenicols (*catB* genes): these genes were prominent plasmid-mediated phenicol resistance. The prevalent genes were *catB3* (28.3%, 31/109), *catB4* (5.4%, 6/109). The overall prevalence of *catB* mediated chloramphenicol resistance was 33.7%(37/109), and *catB* is the only gene identified for phenicol resistance.

Fosfomycin (*fosA*): *fosA* was the only resistance gene and presented at a prevalence of 10.1%(11/109), it is also the less prevalent resistance genes.

Macrolides (*mphA*): the rate of *mphA* detection was 12.8%(14/109), and mphA is the only acquired resistance gene with phosphotransferase property identified in these *K. pneumoniae* strains.

Fluorouinolones (PMQR): the overall prevalence of quinolone resistance gene harboring strains was 94.4%(103/109). Genetic determinants of plasmid mediated quinolone resistance can be classified in to three groups: qnr genes (*qnrA*, *qnrB*, *qnrS*, *qnrC*, and *qnrD*), *qepA*, and *aac(6')-Ib-cr* genes. The aminoglycosides and ciprofloxacin resistance determinant, *aac(6')-Ib-cr* (65.1%), was most prevalent among PMQR genetic factors identified. The other sub-groups of quinolone resistance genes were less frequent *qnrS1* (16.5%, 18/109), *qnrB6* (11.9%, 13/109) and *qepA* (0.9%, 1/109). All *qnrsB6* carrying strains also carry *aac(6')-Ib-cr* genes and *qnrS* genes. From *qnrS* genes 10/18(55.5%) also carry *aac(6')-Ib-cr* genes.

Tetracycline (tet-genes): the tet-genes defined as efflux mediated tetracycline resistance were prevalent. The *tetA* (37.6%, 41/109), *tetD* (18.3%, 20/109), and *tetC* (3.6%, 4/109) with combined overall prevalence of 57.8%(65/109) strains (harboring at least one tet gene).

Sulfonamides (*sul*-genes): genetic factors of resistance for sulfonamides were one of the resistance genes identified with highest prevalence. The overall prevalence of strains with at least one of the *sul* genes was 92/109 (84.4%), where strains with *sul1* gene account for 61.5% and followed by *sul2* that account for 60.5%. There were also strains harboring both *sul1* and *sul2* genes at the same time and the prevalence of these strains was 35/109 (32.1%).

Rifampicin (arr-genes): the rifampicin resistance genes were identified in more than a third of the strains. An overall prevalence of any rifampicin resistance genes was 39.4%(43/109). The prevalence of *arr-2* (7.3%), and *arr-3* (32.1%). No two strains were found to harbor both genes at the same time.

Table 3.18: Genetic determinants of antimicrobial resistance among *K. pneumoniae* strains

S.no	Classes of antimicrobials	Sub-class genes	Genes detected	# strains
1	Aminoglycosides	Acetylation genes	*aac(3)-IIa*	18(16.5%)
			aac(3)-IId	1(0.9%)
		Aminoglycosides and floroquinolone	***aac(6') Ib-cr***	**71(65.1%)**
		Adenylation	*aac(6')-IIc*	2(1.8%)
			aacA4	2(1.8%)
			aadA1	4(3.6%)
			aadA2	14(12.8%)
			aadA5	1(0.9%)
			aadB	3(2.7%)
		Streptomycin	*strA*	**45(41.3%)**
			strB	**45(41.3)**
		Phosphorylation	*aph (3')-Ia*	10(9.2%)
2	β-lactams	*bla*$_{CMY}$	*bla*$_{CMY-6}$	1(0.9%)
			bla$_{CMY-42}$	2(1.8%)
		bla$_{CTX-M}$	*bla*$_{CTX-M-11}$	4(3.6%)
			bla$_{CTX-M-14}$	2(1.8%)
			bla$_{CTX-M-15}$	**92(84.4%)**
			bla$_{CTX-M-3}$	1(0.9%)
			bla$_{CTX-M-9}$	1(0.9)

		bla$_{OXA}$	*bla*$_{OXA-1}$	**38(34.8%)**
			bla$_{OXA-10}$	12(11.0%)
		bla$_{TEM}$	*bla*$_{TEM-1B}$	**76(69.7%)**
			bla$_{TEM-1A}$	2(1.8%)
			bla$_{SHV-1}$	14(12.8%)
		bla$_{SHV}$	*bla*$_{SHV-11}$	23(21.1%)
			bla$_{SHV-26}$	3(2.7%)
			bla$_{SHV-27}$	4(3.6%)
			bla$_{SHV-28}$	7(6.3%)
3	Carbapenem	Carbapenemase	***bla*$_{NDM-1}$**	**1(0.9%)**
4	Phenicols		*catB3*	**31(28.4%)**
			catB4	6(5.4%)
5	Trimethoprim		*dfrA1*	5(4.5%)
			dfrA12	10(9.2%)
			dfrA17	2(1.8%)
			dfrA27	**35(32.1%)**
			dfrA5	1(0.9%)
			dfrA7	14(12.8%)
6	Fosfomycin		*fosA*	11(10.1%)
7	Macrolides		*mph(A)*	14(12.8%)
8	Quinolones	QepA	*qepA*	1(0.9%)
		QnrB	*QnrB6*	13(11.9%)
		QnrS	*QnrS1*	18(16.5%)
8	Tetracycline	Tet	*tet(A)*	**41(37.6%)**
			tet(c)	4(3.6%)
			tet(D)	20(18.3%)
10	Sulfoonamides	Sul	*sul1*	**67(61.5%)**
			Sul2	**66(60.5%)**
11	Rifampicin	ARR	*arr-2*	8(7.3%)
			arr-3	**35(32.1%)**

Key: Bold:most prevalent genes in the categorey

In general, genetic determinants of antimicrobial resistance for eleven different and/or partly related antimicrobial classes were defined with in this collection of *K. pneumoniae* strains. A relative proportion of the genetic factors varies and not all genetic determinants were equally distributed among the strains. However, genetic determinants of resistance for antimicrobials of higher clinical significance were represented with higher proportions, including third generation cephalosporins, aminoglycosides, and fluoroquinolones.

Moreover, a large proportion of these strains encoded multiple different genes of resistance for a given class of antimicrobials. There were about 12 different variants of genetic determinants for resistance to drugs in the class of aminoglycosides.

3.3.4 Multi-Locus Sequence Type (MLST)

MLST is well-known strain typing method, an important epidemiological tool to study long-term epidemiological trends and dissemination of bacterial strains. The Acthman-7 gene MLST (*gapA; infB; mdh: pgi; phoE; rpoB; tonB*) was used for typing of these strains.

MLST was performed on web-based *in silico* bioinformatics tool, **MLST 2.0,** at Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/MLST/. Most strains were assigned a sequence type. However, few strains are novel and yet to be assigned a sequence type (ST).

More than thirty different sequence types were determined and most frequently identified sequence types include:- ST218 (13%, n=7), ST15 (11.1%, n=6), ST17(11.1%, n=6), ST147 (11.1%, n=6), ST39 (9.3%, n=5). Strains with sequence type represented by only a single isolate and sequence type not-defined (novel) were not included in the figure (**Figure 3.10**).

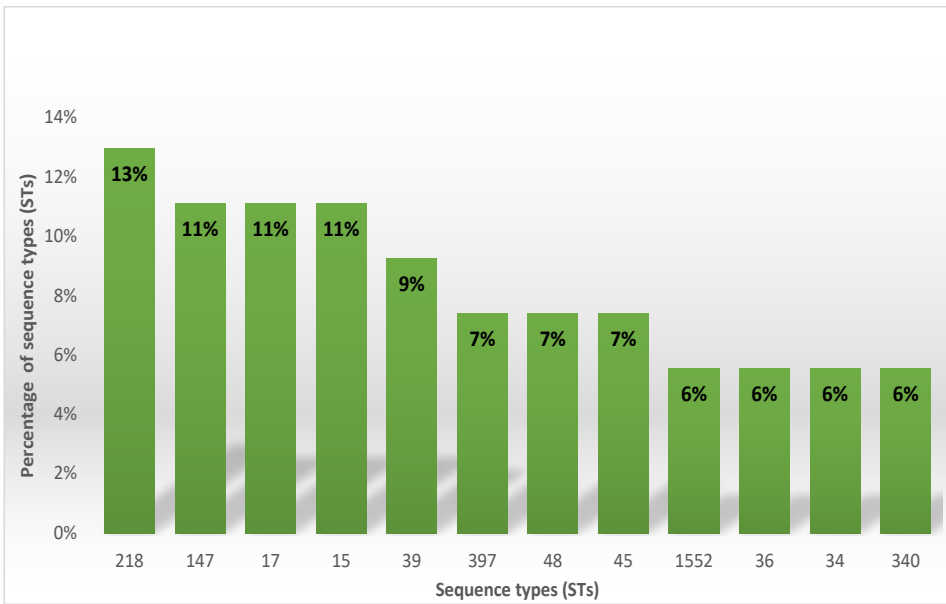

Figure 3.10: **Proportion of ST types among *K. pneumoniae* strains from JMC**

Epidemic clonal groups: several sequence types detected were international clones and also reported elsewhere as epidemic and/or high-risk clones. These clones include ST14, ST15, ST17, ST20, ST147, ST340, and ST101. These epidemic clones account for 22.0% of *K. pneumoniae* strains.

3.3.5 Plasmids and replicon typing

Plasmid replicon typing is one of the molecular features of a bacterial strains important for antimicrobial resistance. The replicon types of plasmids from genome of *K. pneumoniae* strains were analyzed. Plasmid replicon typing was performed by using *in silico*, whole plasmid genome detection and replicon characterization pipeline, Center for Genomic Epidemiology: *PlasmidFinder1.3*,(https://cge.cbs.dtu.dk/services/PlasmidFinder/).

S.no	Replicon types	Freq. (%)	STs (n)
1.	*IncFIB(K/Mar/pQil)*	32(32.9)	ST17(4), ST218(4), ST39(4), ST45(3), ST1552(3), Novel(3), ST34(2), ST66(1), ST514(1), ST551(1), ST685(1), ST297(1), ST552(1), ST530(1), ST54(1), ST664(1)
2.	*IncR*	24(24.7)	ST15(5), ST147(5), ST340(3), ST268(2), ST17(1), ST20(1), ST1800(1), ST390(1), ST14(1), ST70(1), Novel (3)
3.	*IncFII*	14(14.4)	ST397(4), ST1552(3), Novel (3), ST34(2), ST337(1), ST551(1),
4.	*IncQ1*	12(12.4)	ST39(4), ST45(2), ST664(2), ST54(1), ST391(1), ST413(1), Novel (1)
5.	*IncHI(1B/2)*	7(7.2)	ST36(2), ST337(2), ST789(1), ST551(1), Novel (1)
6.	*IncA/C2*	3(3.09)	ST39(1), ST45(1), ST13(1)
7.	*FIA (pBK30683)*	1(1.03)	Novel (1)
8.	*Col (IRGK)*	1(1.03)	ST13(1)
9.	*IncN*	1(1.03)	ST534(1)
Grand Total		**97(100)**	

Table 3.19: Prevalence of plasmids and its distribution among different sequence types

A total of 97 plasmids/replicons were characterized among the *K. pneumoniae* strains. In all except four strains a plasmid genome was identified. However, we considered only plasmid sequences for which the query sequence matches 100% to the reference both in coverage and

percentage identity for analysis. Hence, 72/107 (67.2%) of *K. pneumoniae* strains found to encode for multiple plasmids (**Table 3.19**). Most frequently identified replicon types include, *IncFIB* (*IncFIBK*, *IncFIBMar*, *IncFIBpQil*) accounts for 32.9%(32/97) and *IncR* (24.7%, 24/97) and *IncQ1* (12.4%, 12/97). These plasmids of different replicon types were widely disseminated within several strains that belong to different MLST types. Most plasmids can carry MDR and virulence functions. *IncFIB* replicons were previously described to encode MDR and virulence (http://www.ncbi.nlm.nih.gov/nuccore/AP001918). And also, they are able to transfer these functions between different strains and species. Similarly, *IncQ1* replicons were described as a broad host range plasmids that can carry MDR functions (http://www.ncbi.nlm.nih.gov/nuccore/HE654726). But the *IncQ1*plasmids need a helper to be transferred between strains.

3.3.6 Virulence factors among strains of *K. pneumoniae*

Multiple virulence factors were identified among these *K. pneumoniae* strains. These factors include protectins such as (capsule, lipopolysaccharides (LPS)), siderophores (enterobactin, yersinobactin, salmochelin, and aerobactin), Adhesins (Fim-1 and FimH-3) and allantoin metabolism (*allA*, *allB*, *allC*, *allD*, *allR*, *allS*) (**Table 3.20**).

Protectins: these group of virulence and/or virulence determinant genes were mainly capsules and lipopolysaccharides. Capsules were found in all the strains and there was diversity of the capsular types between the strains. The capsular determinant factors can be classified as determinants of hypermucoviscous and classical mucous phenotype. The hypermucoviscous phenotype determinants identified were mainly *rmpA*/*rmpA2* genes.

Capsule (K-Loci): capsular typing was done based on *cps* (capsular gene complex) analysis on a web-based-tool called Kaptive web (http://kaptive.holtlab.net). A total of 39 different K-loci (capsule types) were identified. Most of the capsular types were found to be the classical capsular types/phenotypes. The KL62 (9.26%, n=10) and KL57 (9.26%, n=10) were most prevalent, and followed by KL25 (6.48%, n=7) and KL64 (6.48%, n=7). From the conventionally hypercapsular types, KL2 (1.85%, n=2) was less prevalent and KL1 was not identified at all (**Table:3-21**).

Table 3.20: **Virulence factors among *bla*$_{CTX-M-15}$ *K. pneumoniae* and functional annotations.**

Category	Comment		Location	Ref.
Adhesins	FimH-1	Type-1 fimbriae/fimA, fimH	Chromosome	(Paczosa and Mecsas, 2016)
	FimH-3	Type-3 fimbriae/mrkABCD	Chromosome	
Protectins	Capsule	Capsule/*rmpA&rmpA2*	Chromosome/plasmid	
	LPs	Lipopolysaccharides	Chromosome	
Siderophores	Enterobactin	Iron chelating factors	Chromosome/plasmid	
	Yersinobactin			
	Salmochelin			
	Aerobactin			
Allantoin Metabolism	*allA*	Allantoin metabolism factors	Chromosome	
	allB			
	allC			
	allD			
	allR			
	allS			
OMPs and Porins	*ompA*	Outer membrane protein A	Chromosome	
	OmpK35	Outer membrane protein of *K. pneumoniae*	Chromosome	
	OmpK36			
Pumps and transporters	*acrAB*	Efflux pump for virulence and resistance	Chromosome	
	kfuA	An ABC transport system	Chromosome	
	kfuB			
	kfuC			

Table 3.20: shows functional annotation of virulence factors extracted from *K. pneumoniae* strains. These virulence factors were described according to putative functions of the virulence factor, description of the virulence factor and possible location in the genome. All virulence factors described above were identified in *K. pneumoniae* strains. However, all strains encode for the type-3 frimbrial antigen, FimH-3. These virulence factors has been also reviewed and summarized in a recent review by Paczosa and Mecsas (Paczosa and Mecsas, 2016).

Table 3.21: **Prevalence of *cps* genes in *bla*$_{CTX-M-15}$ producing *K. pneumoniae* at JMC**.

S.no	K-Loci *cps*-genes	Prevalence (%)	STs(n)
1.	KL62	9.2%	ST39(4), ST48(4), ST664 (2)
2.	KL57	9.2%	ST218 (8), ST13 (02)
3.	KL64	6.5%	ST147(6), Novel (2)
4.	KL25	6.5%	ST17(3), ST1552(3), ST45(1)
5.	KL158	5.6%	ST397(4), Novel (02)
6.	KL24	4.6%	ST15(5)
7.	KL3	4.6%	ST1107(02), ST394(1), ST14(1), ST896(1)
8.	KL27	3.7%	ST36(2), ST685(1), Novel (1)
9.	KL107	3.7%	ST36(02), ST534(1), Novel (1)
10.	KL15	2.8%	ST340(3)
11.	KL119	2.8%	ST340(3)
12.	KL20	2.8%	ST268(2), ST45(1)
13.	KL155	2.8%	ST17(3)
14.	KL10	2.8%	ST551(1), ST297(1), Novel (1)
15.	KL117	1.9%	Novel (2),
16.	KL30	1.9%	ST198(1), ST391(1)
17.	KL122	1.9%	ST70(1), Novel (1)
18.	KL23	1.9%	ST39(1), Novel (1)
19.	KL52	1.9%	ST45(2)
20.	KL110	1.9%	Novel (02)
21.	KL54	1.9%	ST530(1), Novel (1)
22.	KL8	1.9%	ST252(2)
23.	KL109	1.9%	ST337(1), Novel(1)
24.	KL2	1.9%	ST15(2)
25.	*Others	13.9%	
Grand Total		**100%**	

*Others: 15 strains (0.9% each)

Table 3.21. shows relative distribution of capsular types among *K. pneumoniae* strains based on the latest classification system proposed by Kaptive (http://kaptive.holtlab.net). Kaptive is a web-based method for running Kaptive and visualizing results. The confidence level of the output can be interpreted as: (i) Perfect: the locus was found in a single piece (one alignment within a single contig) with 100% coverage and 100% nucleotide identity to the reference, (ii) Very high: the locus was found in a single piece with ≥99% coverage and ≥95% nucleotide identity to the reference, with no truncated/missing genes and no extra genes compared to the reference, (iii) High: the locus was found in a single piece with ≥99% coverage, with ≤3 truncated/missing genes and no extra genes compared to the reference, (iv) Good: the locus was found in a single piece or with ≥95% coverage, with ≤3 truncated/missing genes and ≤1 extra gene compared to the reference, (v) Low: the locus was found in a single piece or with ≥90% coverage, with ≤3 truncated/missing genes and ≤2 extra genes compared to the reference, (vi) None: did not qualify for any of the above. The regular capsular type designation "K1, K2...etc." was replaced by capsular locus (KL), and hence, KL1, KL2…etc., in Kaptive system.

Regulators of mucoid phenotype (*rmpA* and *rmpA2*): acquired genes that has been long characterized as determinants of hypermucoid phenotype (regulators of mucoid phenotype). These genes render bacteria a hypercapsule phenotype. The *rmpA* and *rmpA2* genes were detected in 8.3% of the strains. These genes were identified predominantly in two MLST types ST218 (8/9) and ST198 (1/9). The association of *rmpA*/*rmpA2* genes to ST218 *K. pneumonie* strains with KL57-capsular and O2v2-lipopolysacharide variants was statistically significant. Another strain ST198-KL30-O1v1 also encoded the *rmpA*/*rmpA2* genes (**Table 3.22**).

Table 3.22: **Unique features of *rmpA*/*rmpA2* encoding *bla*$_{\text{CTX-M-15}}$ strains**

S.n	Strain ID	diagnosis	Unit	presence of *rmpA* genes	K-Loci	O-LPS	MLST	Fisher exact test P-value < 0.0001
1	M079kp	Pneumonia	Admitted	*rmpA*/*rmpA2*	KL57	O2v2	218	
2	M228kp	UTI	Out-patient	*rmpA*/*rmpA2*	KL30	O1v1	198	
3	P049kp	Diarrhea	Admitted	*rmpA*/*rmpA2*	KL57	O2v2	218	
4	P051kp	SCAP	Out-patient	*rmpA*/*rmpA2*	KL57	O2v2	218	
5	P146kp	Diarrhea	Admitted	*rmpA*/*rmpA2*	KL57	O2v2	218	
6	P156kp	Diarrhea	Admitted	*rmpA*/*rmpA2*	KL57	O2v2	218	
7	S149kp	SSI	Admitted	*rmpA*/*rmpA2*	KL57	O2v2	218	
8	S244kp	WI	Admitted	*rmpA*/*rmpA2*	KL57	O2v2	218	
9	S316kp	WI	Admitted	*rmpA*/*rmpA2*	KL57	O2v2	218	

COPD: chronic obstructive pulmonary disease, UTI: urinary tract infection, SCAP: severe community acquired pneumonia, SSI: surgical site infection, WI: wound infection, NF: necrotizing fasciitis.

Lipopolysaccharides (O-antigens): outer membrane component of Gram-negative bacilli contains lipopolysaccharides (LPS). One of the important components of polysaccharides for bacterial pathogenesis, the O-antigen was characterized for the *K. pneumoniae* strains.

A total of twelve different O-antigen types were identified, mainly: O1v1, O1v2, O2v1, O2v2, O3, O4, and O5 from the classical types, and OL101, OL102, OL103 from novel types. Overall, O1 (53.7%, n=54) and O2(23.15% n=25) were most prevalent types and other less frequent types were also detected **(Table 3-23)**

Table 3.23: Prevalence of O-LPS among ESBL-producing *K. pneumoniae*

S.no.	O-LPs Loci	O-Loci (%)	K-loci (n)
1.	O1v1	31.5%	KL62(6), KL158(6), KL24(5), KL25(3), KL117(2), KL10(2), KL2(1), KL17(1), KL18(1), KL30(1), KL39(1), KL64(1), KL68(1), KL81(1)
2.	O1v2	22.2%	KL62(4), KL3(3), KL8(2), KL57(2), KL54(2), KL122(2), Kl108(1), KL109(1), KL11(1), KL112(1), KL136(1), KL2(1), KL23(1), KL28(1), KL122(1)
3.	O2v2	12.9%	KL57(8), KL3(2), KL27(2), KL102(1), KL109(1)
4.	O2v1	10.2%	KL64(6), KL20(2), KL110(2), KL25(1)
5.	O4	8.3%	KL119(3), KL141(1), KL15(3), KL27(2)
6.	OL101	7.4%	KL155(3), KL107(2), KL52(2), KL103(1),
7.	O5	2.8%	KL25(2), KL10(1),
8.	OL104	0.9%	KL25(1)
9.	OL103	0.9%	KL23(1)
10.	O3/O3a	0.9%	KL20(1)
11.	O3b	0.9%	KL14(1)
12.	OL102	0.9%	KL107(1)

Table3.23 prevalence of O-LPS and distribution of O-lipopolysaccharides among KL-locus show greater diversity. However, O1 and O2 variants were the most prevalent O-LPS. The novel O-LPS like OL-102, and OL103 were identified. Based on Kaptive a web-based typing tool, the O-typing was performed using the whole genome sequence data. Many novel and classical O-lipopolysaccharides were identified. The O1 and O2 and their variants often serologically cross reactive were prevalent.

Adhesins/invasion determinant genes:

Adhesins are one of the important determinant phenotypes for bacteria to attach to both abiotic and biotic surfaces. Nearly all of the strains encoded for both type-1, and type-3 fimbriae. All strains that encoded fimbrial antigens were definded by presence of complex gene cluster *(fimA*, *fimB, fimC*, *fimD*, *fimH, fimI*) for type-1, and the complex gene cluster (*mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkF*, *mrkI*, *mrkH*, *mrkJ*) for type-3.

Genetic determinants of siderophores:

Iron scavenging is a major strategy for *K. pneumoniae* survival in the host tissue. There are several mechanisms that bacteria use to compete for iron from the hosts system. Three main genetic determinants of iron scavenging factors (yesinobactin, salmochelin and aerobactin) were identified. The prevalence of these genetic determinants varied from 6.5% - 42.5%.

Yersinobactin: strains comprising yersinobactin genetic determinant locus were identified as *irp1* (7/108), *irp2* (7/108), and *ybtQ* (46/108).

Salmochelin: yersinobactin are another type of siderophores found in these strains. The genetic determinant for salmochelin iron acquisition system, *iroBCDE* gene cluster were detected in 8/108 (7.4%) of the strains.

Aerobactin: the *iucABCD* gene cluster for aerobactin and the aerobactin transporter *iutA* were carried on the genomes of 10/108 (9.2%) of strains.

Miscellaneous virulence genes in *K. pneumoniae* strains

Allantoin metabolism genes: allantoin metabolism, a way by which a bacterium obtains carbon and nitrogen from it's environment. A genetic determinant of metabolism of allantoin were detected in 5 (4.6%) of strains.

Outer membrane proteins (OMPs): outer membrane proteins are an important structural and functional unit of outer membrane and belived to be associated with virulence of a bacteria. Both presence and absence of these structures and functions influences fitness and virulence of a strain. All strains in this collection studied for outer membrane protein A (*ompA*), and porins (*ompK35* and *ompK36*) did not encode these genes on preliminary screening, however these findings must be evaluated further.

Pumps and transporters: the *Kfu*-ABC transport system used for acquisition of iron by *K. pneumoniae* were identified in 35(32.4%) of strains. The most commonly known, and well characterized chromosomal mediated multidrug resistance efflux pumps, *AcrAB* system (*AcrB*, and *AcrR*) were identified in most of these strains.

3.3.7 Polyclonal population structure of strains

A phylogenetic analysis of *K. pneumoniae* strains demonstrated that several of the international clones detected were clonally clustred. The most common clusters include: ST17, ST15, ST39, ST48, ST218, ST147, ST397 and ST34. Showing the dissemination of $bla_{\text{CTX-M-15}}$ genes was through polyclonal, both international and local clones. The dissemination was not restricted to a given unit, and these strains were identified at medical, surgical, and pediatric units in the hospital, also from the different sample types. (**Figure 3-11**).

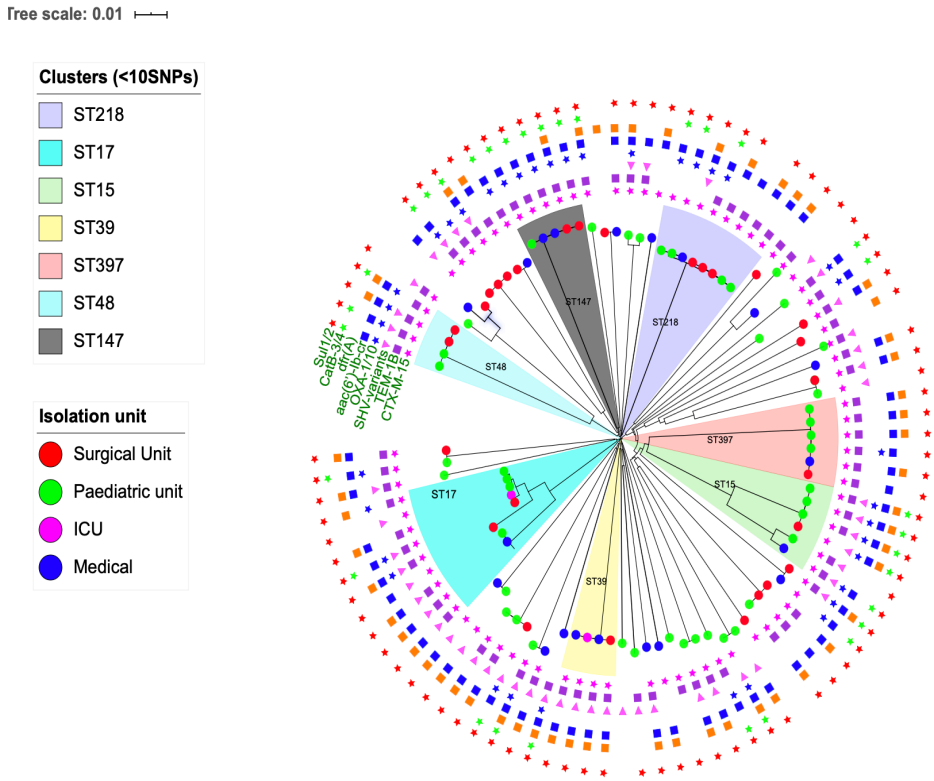

Figure 3.11: Polyclonal clusters of *bla*$_{\text{CTX-M-15}}$ producing *K. pneumoniae* strains at JMC

Furthermore, these clusters were associated to multiple antimicrobial resistance genes. Strains in a given clade seemingly harbor similar genetic determinant profile (**Figure 3.12**). The genetic profile of antimicrobial resistance genes includes *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{SHV}}$-variants, *bla*$_{\text{OXA}}$-variants, *aac(6')-Ib-cr*, *sul1*& *sul2*, *dfrA*-variants, *tet*, and *cat* genes (**Figure 3.12**)

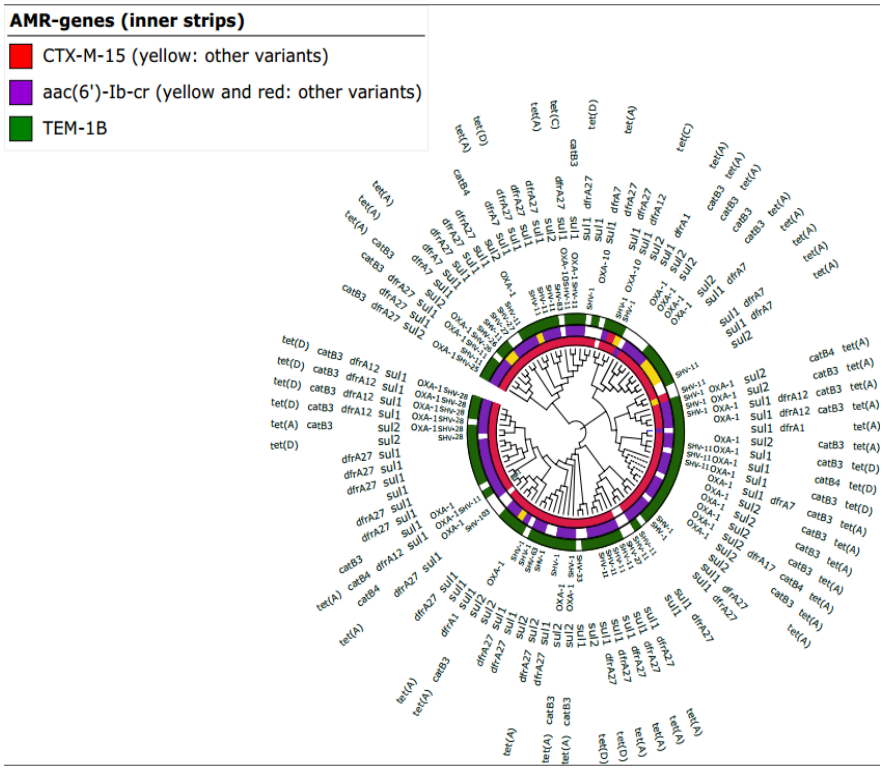

Figure 3.12: *bla*$_{\text{CTX-M-15}}$ producing *K. pneumoniae* clones encoding multiple resistance gene

The figure shows clonal structure of ESBL producing *K. pneumoniae* strains and antimicrobial resistance genes. The AMR genes include β-lactamases (*bla*$_{\text{CTX-M}}$-types, *bla*$_{\text{SHV}}$-types, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{OXA}}$-types,), Aminoglycosides (*aac(6')-Ib-cr*, *aac(3)IIa*, *aadA2*), sulfonamides (*sul1* and *sul2*), trimethoprim (*dfrA27*, *dfrA12*, *dfrA7, dfrA1*), phenicol's (*catB3* and *catB4*), tetracycline (*tetA*, *tetD* and *tetC*). In this figure, the most inner strip represents *bla*$_{\text{CTX-M}}$-types (Red - *bla*$_{\text{CTX-M-15}}$, Purple-*bla*$_{\text{CTX-M-14}}$, and Yellow-*bla*$_{\text{CTX-M-1}}$). The second most inner strip represents aminoglycosides (Purple: *aac(6')-Ib-cr*, Yellow: *aac(3)IIa*, and Red: others). The third, outer-most strip represents *bla*$_{\text{TEM}}$- types (Green: *bla*$_{\text{TEM-1B}}$).

CHAPTER 4: DISCUSSION

4.1 *E. coli* strains

Infections caused by MDR strains of *Enterobacteriaceae* pose a challenge to medical care and public health. The increasing failure of broad-spectrum antimicrobials hinder empirical therapy for complicated and/or serious infection that demand immediate clinical intervention. *E. coli*, one of the most frequently isolated *Enterobacteriaceae* from clinical specimens often from serious infections including infections to bloodstream and other sterile sites in the body has developed resistance to several antimicrobials.

4.1.1. Prevalence: the rate of ESBL among *E. coli* strains isolated in the current study is 54.9%. Some of these ESBL strains were non-susceptible to carbapenem, however, only two of the strains encoded carbapenemase gene, and the reduced susceptibility of other strains to carbapenems might be a non-carbapenemase mediated resistance to carbapenems.

In this study, a higher prevalence of ESBL-producing strains was determined as compared to previous phenotypic studies from the same hospital (Mulualem *et al*., 2012; Moga *et al*., 2014). These previous studies were conducted on a relatively smaller sample size, and over a short period of time. Thus, lower sample size might have contributed to the lower prevalence of the ESBL-producing strains in those studies. Most importantly, there is a palpable time gap between the current study and those previous studies, it might be possible that the rate of ESBL is rising in the hospital over these years.

Studies from other African countries like Mali, Bamako, reported a very high prevalence of ESBL in *Enterobacteriaceae* and this finding is comparable to the rate of ESBL in our study (Sangare *et al*., 2017). In one of the non-systematic reviews on data from Africa, different rates of ESBL were reported in many countries including Egypt, Morocco, Algeria, Guinea Bissau, Kenya and others (Storberg, 2014). In many of the cases, prevalence of ESBL-producing *Enterobacteriaceae* in these countries was lower than rate of ESBL reported in the current study. However, most of the studies covered by the review were studies with limited sample size and the majority of these studies used non-genetic methods of detection.

Though the prevalence of ESBL-producing *E. coli* strains is lower in USA and Europe, several studies reported increasing prevalence of ESBL-producing strains (Arnaud *et al*., 2015; Canton

et al., 2008). As compared to global, the prevalence of ESBL is very low in Europe. However, Eurosurveillance report shows that there were regions where the incidence of ESBL was increasing from 3% to 5% per 100,000 patient-days (Arnaud *et al*., 2015). A retrospective cohort study conducted on 36 hospitals in the USA reported that the incidence of ESBL-producing *E. coli* increased from 5.2% to 10.5% per 100,000 patient-days over a period of five years. Moreover, the number of hospitals reporting ESBL drastically increased over six years (2009-2014) from community hospital in the south eastern part of USA (Thaden *et al*., 2016).

In Asia, there were several reports of very high prevalence of ESBL-producing *E. coli* strains. One of the recent reviews on epidemiology of MDR *E.coli* strains reported that *E. coli* strains producing $bla_{\text{CTX-M}}$ enzymes reached 60%-79% (Sidjabat and Paterson, 2015). According to this review and studies described elsewhere, it seems that Asian continent remains to be hotspot of antimicrobial resistance. The prevalence of $bla_{\text{CTX-M-15}}$ ESBLs and rate of AMR in Africa and specifically Ethiopia is not less than the Asian countries. The difference might be that the burden of AMR was not well studied in African countries. When it is studied like a study from Bamako, it is as high as 76.9%, and we also report here a higher prevalence of ESBL in *E. coli*.

Another important resistance trait of clinical strains is carbapenem resistance. In this study, only 5.4% of ESBL producing *E. coli* strains were non-susceptible to carbapenem, and two of the carbapenem non-susceptible strains encoded carbapenemase genes $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-66}}$. Carbapenem susceptibility of these strains is an important information for local consumption. Nevertheless, use of carbapenem should be considered for treatment of infections from ESBL strains under strict regulation to prolong utility of these drugs in the hospital. Similarly, other parts of the world where carbapenem is commonly used, carbapenemase mediated carbapenem resistance emerged correspondingly.

On the other hand, these *E. coli* strains were MDR strains, showing resistance to several classes of antimicrobials commonly prescribed for infections caused by *Enetroabcetriaceae* strains. All of these ESBL-strains of *E. coli* were resistant to at least two class of antimicrobials and most notably 52% of them were resistant to five classes of antimicrobials that include: trimethoprim, aminoglycosides, fluoroquinolones, and piperacillin-tazobactam. Several other studies have also reported MDR-ESBL *E. coli* strains. The challenge in the increased rate of

MDR remains pronounced, if carbapenems should be spared (Docquier and Mangani, 2018; Pranita and Rodriguez-ba, 2017).

The increased use of carbapenems may lead to carbapenemase mediated carbapenem resistance that may end up at a foreseeable compromised therapeutic arsenal and impose major challenge on clinical care and public health (Bevan *et al*., 2017). WHO recommended surveillance of infected and asymptomatic carriers of carbapenemase-producing strains and where necessary instituting strict infection control policy/hygiene may reduce burden of antimicrobial resistance in low-income countries (WHO, 2017).

4.1.2. Genetic determinants of resistance: several genetic determinants of antimicrobial resistance were detected among these strains. Predominant ESBL types were class A-ESBLs. All strains bear *bla*$_{\text{CTX-M}}$ genes: *bla*$_{\text{CTX-M-15}}$ (88.4%), *bla*$_{\text{CTX-M-14}}$ (4.3%), *bla*$_{\text{CTX-M-11}}$ (2.8%), *bla*$_{\text{CTX-M-27}}$ (2.1%) and *bla*$_{\text{CTX-M-55}}$ (0.7%). A difference in rate of detection between different *bla*$_{\text{CTX-M}}$ variants is considerable, the high prevalence of ESBLs is mainly because of *bla*$_{\text{CTX-M-15}}$ variants.

In global perspective, *bla* $_{\text{CTX-M-15}}$ is the most prevalent genetic determninant of ESBL. In a recent review of global epidemiology of *bla*$_{\text{CTX-M,}}$ many parts of African countries reported to have *bla*$_{\text{CTX-M-15}}$ variants. The Asian countries reported mainly *bla*$_{\text{CTX-M-14}}$, and south American countries reported another variant, *bla*$_{\text{CTX-M-2}}$ (Bevan *et al*., 2017). The data has revealed differences in prevalence and geographical distribution of *bla*$_{\text{CTX-M}}$ variants. Emerging studies form Africa are reporting high prevalence of *bla*$_{\text{CTX-M-15}}$-producing *Enterobacteriaceae*, mainly among *E. coli* strains from both clinical and non-clinical niches (Moremi *et al*., 2017; Ouedraogo *et al*., 2016; Ribeiro *et al*., 2016). Such evidence, together with lack of strict antibiotic policy and infection control strategies, and lack of enough alternative antimicrobials is a clear challenge to the region. This study calls for an urgent and tailor-made interventions.

Furthermore, these strains encoded for genetic variants of other β-lactamases which include *bla*$_{\text{OXA}}$, *bla*$_{\text{TEM}}$, and *bla*$_{\text{SHV}}$ types. The second most prevalent β-lactamase genetic determinant is *bla*$_{\text{OXA-1}}$ (63.3%), other *bla*$_{\text{OXA}}$ variants are rare. The *bla*$_{\text{TEM-1B}}$ (53.9%) another β-lactamase and the only genetic variant of *bla*$_{\text{TEM}}$ type ESBLs was identified in majority of strains.

Whereas, *bla*$_{SHV}$ variants were not common in *E. coli* strains and *bla*$_{SHV-12}$ detected in only one of the strains.

Besides, two distinct but related pAmpC β-lactamases (*bla*$_{CMY-2}$ (12.2%) and *bla*$_{CMY-42}$ (1.4%)) were also identified. ESBLs were practically defined as MDR strains by their own, however, ESBL strains were found to bear other co-resistance genes including carbapenemases, other-β-lactamases and non-β-lactamases resistance genes. As it has been described above *E. coli* strains isolated in the current study carry several other genetic determinants of resistance also to drugs of non-β-lactam classes. Generally, there were several other studies that reported a similar phenotypic and genotypic features of such clinical isolates from different parts of the world (Lutgring and Limbago, 2016; Ouedraogo *et al*., 2016; Sidjabat and Paterson, 2015).

A very high prevalence of co-resistance of ESBL strains with several other narrow-spectrum β-lactamases largely limits the use of non-carbapenem β-lactam therapeutic options, and β-lactam and β-lactam inhibitors combinations for treatment of infections caused by ESBL-producing *E. coli* strains. The β-lactam-β-lactam inhibitors combination is considered as promising alternative treatment options for ESBL-producing strains as carbapenem sparing alternative (Angelo *et al*., 2016; Docquier and Mangani, 2018; Sharma *et al*., 2016; Pranita and Rodriguez-ba, 2017). Several of β-lactam-β-lactam inhibitors combinations are currently approved and many more are being investigated (Docquier and Mangani, 2018). The use of β-lactam-β-lactam inhibitors combinations will be compromised when ESBL strains co-produce other multiple β-lactamases and may also have other resistance mechanisms.

Effectiveness of these therapeutic alternatives were primarily dependent on several other factors including inoculum effect, dosing or scheduling, pharmacokinetic/pharmacodynamic parameters, and other patient specific conditions including severity of a disease. Moreover, when the β-lactam-β-lactam inhibitors are scavenging the co-existing narrow spectrum-β-lactamase abundant in that microenvironment, outcome of treatment might be severely challenged and the consequences might be worse (D'Angelo *et al*., 2016; Sader *et al*., 2007; Sharma *et al*., 2016; Pranita and Rodriguez-ba, 2017).

Also, the prevalence of resistance to non-β-lactam antimicrobials is high. Multiple and high prevalence of resistance determinant genes for aminoglycosides, sulfonamides, phenicols,

trimethoprim, and macrolides were detected. The multiple resistance to several antimicrobials commonly prescribed at the site signals clear danger. Prescribers, policy makers, and all other stakeholders should consider taking an immediate action.

Most importantly, genetic determinants aminoglycosides and fluoroquinolones (*aac(6')-Ib-cr* are highly prevalent among these ESBL strains. This co-resistance feature of ESBLstrains of *E. coli* has also been reported from studies elsewhere, both from strains of human and animal origin, revealing that it is spreading across a wider niche (Kim *et al*., 2011; Ojdana *et al*., 2018; Seni *et al.*, 2016; Tsukamoto *et al*., 2014). And yet, the *aac (6')-Ib-cr* gene was a main genetic determinant of plasmid-mediated aminoglycoside and fluoroquinolone resistance in this study. Co-existence of $bla_{\text{CTX-M-15}}$ with three genetic determinants of aminoglycoside resistance was statistically significant. These are *aac(6')-Ib-cr* (p-value = 0.006), *aadA5* (p-value = 0.028), and *aadA2* (p-value = 0.000). It might be possible that these genes have been co-transmitted on the same plasmid or same transferable genetic material. Several other investigators, including studies conducted on genetic analysis of plasmid reported the co-occurrence of *aac(6')Ib-cr* genes with $bla_{\text{CTX-M-15}}$ on the same mobile genetic element (Bado *et al*., 2016; Dolejska *et al*., 2012; Guillard *et al*., 2014; Silva-Sánchez *et al*., 2013).

4.1.3. Plasmids: Fifteen plasmid replicon types (*Inc FII, IncQ1, Col (BS512), IncX4, IncI1, IncFII, IncA/C2, IncHI2A, IncI2, IncHI2, IncB/O/K/Z/, IncR, IncFIB (AP001918), IncFIA, IncFIB(pQil)*) were detected among $bla_{\text{CTX-M-15}}$-producing *E. coli* strains. Strains harboring *IncFII (pRSB104)* (33%) were most prevalent, followed by *IncQ1* (27%), and *Col* (BS512) (10%). *IncFII* plasmids were the most commonly identified replicon types associated with $bla_{\text{CTX-M-15}}$. In a review by Caratolli, *IncFII* plasmids were reported to encode and carry several resistance genes including $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, and *acc(6')-Ib-cr*, and are highly conserved low copy number plasmids (Carattoli, 2009). It is possible that these strains might have acquired these genes on the same plasmid by the same mechanism.

On the other hand, high rate of *IncF* replicons were reported in strains collected from different sources like drinking water, pit latrine, and animal origins (Carattoli, 2009; Marcade *et al*., 2009; H. Zhang *et al*., 2016). With these properties, *IncF* plasmids and other similar prevalent replicon types might be responsible for the dissemination of $bla_{\text{CTX-M-15}}$ and other resistance

genes. Provided that the most prevalent plasmid replicon in our study among *bla*$_{\text{CTX-M-15}}$ strains was *IncF* families (*IncFII*) and might also encode for multiple resistance genes including (*bla*$_{\text{TEM-1,}}$ *bla*$_{\text{OXA-1}}$ and *aac(6')-Ib-cr)*; it is highly likely possible that the dissemination of genetic determinants of MDR in the hospital is mainly through *IncF* replicons.

The *IncQ1* plasmids were second most prevalent replicons in *bla*$_{\text{CTX-M-15}}$-producing *E. coli* strains in this study. *IncQ1* was characterized as non-conjugative, mobilizable and broad-host range plasmid (Loftie-Eaton and Rawlings, 2012; Zhao and Hu, 2013). There are studies that reported *IncQ1* plasmids carrying MDR genes among salmonella strains. Another study conducted on swine farm reported *IncQ1* carrying *IMP-27* carbapenemase gene among *E. coli* strains (Mollenkopf *et al*., 2017). However, *IncQ1* plasmids were not reported from ESBL-producing strains. Studies regarding the role of *IncQ1* in dissemination of *bla*$_{\text{CTX-M-15}}$ were limited. However, the ecological overlap (co-existence of several different species) and biological overlap (the functions of two or more plasmids may co-exist in a bacteria and genes may jump from one to the other) makes it putative factor for dissemination of several multiple genetic determinants of resistance genes. We report *IncQ1* plasmids among strains isolated from stool specimens and predominantly from ST410 *E. coli* strains seemingly dissemination within a confined source. Detail characterization of the genetic environment of the plasmids and ST410 strains harboring these plasmids need to be addressed in the future. Also, the role of *IncQ1* plasmids in dissemination of *bla*$_{\text{CTX-M-15}}$ may need further attention. To the knowledge of these investigators, this study is the first to report *IncQ1* associated with *bla*$_{\text{CTX-M-15}}$ encoding *E. coli* strains from human clinical specimens.

Similarly, other replicon types detected in our study can carry MDR functions and capable of transferring the genetic elements. Some of them were broad-host plasmids, and some of them were narrow-host. These plasmids were either conjugative or mobilizable, and hence, the presence of these plasmids can be accounted for the dissemination of ESBL-genes among the bacterial population at the study area.

4.1.4.Virulence and pathotypes: because most species that belong to *Enterobacteriaceae* reside in a gut of human and animals, these strains are usually considered as commensals. They account for major portion of the gastrointestinal flora, and major portion of organisms often

studied as gut microbiota. However, some strains of *E. coli* are pathogenic and encode distinct virulence genes. These virulence genes can be acquired either through plasmid mediated genetic transfer or chromosomally encoded (Cabal *et al*., 2016). We have studied prevalence of virulence genes among ESBL-producing *E. coli* strains. Majority of strains 72(51.7%) bear at least one virulence genes, and 26 (18.7%) of these strains carry three to nine virulence genes.

Presence of these virulence genes is related to a characteristic pathological feature. Based on these pathological features *E. coli* can be grouped in to several pathotypes. Most commonly studied pathotypes include: EPEC, EHEC, EIEC, EAEC, DAEC, EHEC and DEC (Cabal *et al*., 2016; Lima *et al*., 2013; Wang *et al*., 2017). Among these strains, we identified 24 different genetic determinants of virulence. These virulence genes were grouped in to five functional categories as: adhesins/adherence factors, toxins, protectins/factors that increase the survival advantage of the strains, siderophores/iron scavenging molecules, invasins and miscellaneous group of virulence factors (**Table 5-14**). Though, several virulence factors have been identified among *E.coli* strains, the presence of one or more specific subset of virulence genes defines an isolate to certain category of pathotype (Croxen *et al*., 2013; Karisik *et al*., 2008; Olesen, 2017).

Several genes related to virulence factors of ExPEC/EPEC (*papA/papC, kpsM II, IutA, afa, pic, iss, ireA, iha, cnf1, sat, iroN and senB*) were identified in majority of the strains. However, a set of virulence genes that define ExPEC were the presence of ≥2 *papA*, and/or *papC* (both counted as one), *sfa/foc*(S and F1C fimbriae), *afa/dra* (Dr-binding proteins), *KpsM II*, (group II capsules) and *iutA* defines ExPEC strains (Croxen *et al*., 2013; Olesen, 2017). Most of the strains were EPEC, and ExPEC/UPEC pathotypes.

EAEC strains were also characterized by the presence of either one or more of *aggR*, *aatA*, *aaiC* virulence genes, that account for 12.9%. All of the EAEC strains were isolated from diarrheic pediatric population. Similarly, high prevalence of EAEC were reported as a cause of both acute and persistent diarrheal disease from studies conducted elsewhere (Croxen *et al*., 2013; *Huang et al*., 2006; Jønsson *et al*., 2015; Lima *et al*., 2013). A comprehensive surveillance birth cohort study (MAL-ED) reported that EAEC strains can also be isolated from children without diarrhea (absence of overt diarrhea), that the infection can be asymptomatic and related to short falls in the growth of children in early life (Rogawski *et al*., 2017).

The objective of this study is not to determine the impact of EAEC strains on malnutrition, but as one of care centers, malnourished pediatric population with diarrhea admitted to the hospital were included in the study, we report the detection of EAEC strains exclusively study subjects with malnutrition. Similarly, a study from Brazil, reported EAEC from diarrheic children with malnutrition. EAEC pathotype was defined based on pathotype defining virulence genes similar to a study from Brazil. In these studies, pathotype defining virulence genes were *aatA* and *aaiC*, and EAEC strains were associated to malnutrition. The study from Brazil further described, the presence of additional virulence genes encoded by EAEC, the additional genes influence the outcome differently, and in some of cases how combination of certain virulence factors are related to malnutrition (Havt *et al*., 2017). The epidemiology of EAEC is unique as compared to other DEC strains. The EAEC strains are diverse, host susceptibility and host response might be heterogenous and occurs over a wider geographic area and different ecologic niches (Estrada-Garcia *et al*., 2014).

Now, when prevalence of antimicrobial resistance is increasingly being reported from several strains; few evidences are available with regard to antimicrobial resistance and treatment alternatives for EAEC, several of the available studies focused on the prevalence, virulence, and pathology of the strain. Ciprofloxacin and fluoroquinolones are drugs of choice for treatment of EAEC, however, previous study reported resistance to some of the drugs like quinolones (Glandt *et al*., 1999). ESBLs were also reported from these strains (Franz *et al*., 2015). In this study, we report EAEC strains that encode for $bla_{\text{CTX-M-15}}$ and multiple virulence factors. These strains encoded multiple resistance genes for several classes of antimicrobials including fluoroquinolones, aminoglycosides, and sulfonamides. On the contrary, study from Banglades has reported there was no concordant relationship between antimicrobial resistance and of virulence genes (Chattaway *et al*., 2017).

Mostly *E. coli* strains encode virulence genes that are related to a particular pathotype, but not all virulence genes are pathotype defining. Some of the EAEC strains identified in this study encoded several different virulence genes. It might be the case of pooling (hybrid) of several virulence genes as it has been characterized for hybrid pathotypes because of plasmid mediated horizontal genetic transfer.

Some studies proclaim that EAEC is an emerging MDR pathogen for which the mechanism of pathogenesis is largely unknown including adhesion, enterotoxin, cytotoxin secretion and cause of mucosal inflammation (Havt *et al*., 2017; Kong *et al*., 2015; Raju and Ballal, 2009). These studies recommended an in-depth study on the molecular mechanisms of resistance of this particular pathotype.

4.1.5. Serotyping: serotyping was one of the methods employed to rapidly detect outbreaks of several pathotypes of *E. coli* strains: O25:H5 ExPEC, O157:H7& O103:H21 STEC, and O104:H4 EHEC strains. The outer membrane of *E. coli* is composed of lipopolysaccharides (LPS) that includes lipid A, core oligosaccharides, and a unique polysaccharide, referred to as the O-antigen. Loss of O-antigens resulted in attenuated virulence, suggesting importance of O-antigen in host-pathogen interactions. *E.coli* strains demonstrate heterogeneity with respect to serotypes and the prevalence of serotypes that vary from place to place (Croxen *et al*., 2013; Fratamico *et al*., 2016; Olesen, 2017).

Serotype prediction was performed with web-based *in silico* prediction model. These strains were serotypically heterogeneous, grouped in to 23 different O-antigen types, six non-typeable, and 52 different O:H combinations. The most prevalent serotypes were O8(27%), O89(14%), O25(6%), O2(6%), O102(6%), O9(4%) and O176(4%). Serotypes O25 and O15 strains were associated with presence of multiple virulence genes. Similarly a study conducted in Mexico, O25, O15 and O8 serogroups were found to be associated with presence of virulence genes (Paniagua-Contreras *et al.*, 2017). The O8 is most prevalent in the study, however, only one strain was found to carry multiple virulence genes. Three serotypes O25, O176 and O15 were strains related to presence of multiple virulence factors. Neither typical serogroups characterized as epidemic/outbreak strains O157:H7, O104:H4, O103:H2, nor virulence genes defining these particular serogroups were identified (Olesen, 2017; Paniagua-Contreras *et al*., 2017).

The epidemiological serogroup related to extra-intestinal infections and commonly called ExPEC, O25:H4 was identified. Strains in these group were found to encode several virulence genes in addition to those ExPEC defining. In fact some of the ExPEC defining virulence genes are chromosomally encoded genes (Paniagua-Contreras *et al*., 2017), and reliability of

serotyping can be questioned in the era of genome sequencing. Furthermore, as investigative techniques and technologies are advancing, novel mechanisms of disease causation, evolution of diagnostic tools, and discovery of new knowledge is pragmatic. Hence, O25:H4 and other serotype strains may heve encoded several virulence genes beyond the conventional virulence defining genes and now detected due to the ultra high resolution of whole genome sequencing (virulence matrix data not shown). Moreover, evolving/developing serotypes/pathotypes being discovered and novel findings are expected. One of such evolutions of hybrid pathotype caused an outbreak in Germany, a hybrid strain of EAEC and STEC, O104:H4. This strain is basically an EAEC pathotype that acquired *shiga-toxin* coding genes (Croxen *et al*., 2013; Olesen, 2017; Rahal *et al*., 2015).

Though these *E. coli* strains were grouped into several different serogroups and some are associated to diseas and others are not, most of them encode virulence genes that enable them colonize tissues in the body and also encode genetic determinants of multiple antimicrobial resistance.

4.1.6. MLST and Epidemiologic strains:diversity in bacterial strains can be determined at different level by using phenotypic and genotypic methods. Phenotypic features may include colony characteristics, biochemical properties, and serological properties that scientists used to classify bacteria into different genuses, species and strains. The phenotypic approach of classifying and identifying bacteria species served over several decades, and yet it is important tool in microbiology. However, with the advent and advance of molecular biology techniques and increased understanding of genomics, genotypic features have become more accurate. Genomics become a main tool for understanding similarity and diversity of bacterial strains. Nowdays, several tools were developed for bacterial typing with higher resolution for outbreak detection and tracking, or epidemiological spread and investigation. MLST typing is one of most commonly used genotyping methods for an epidemiologic investigation.

Over 25 different sequence types were identified, and most commonly identified sequence types were ST410 (23%), ST648 (17%), ST131 (10%), ST10 (9%), ST2659 (8%), ST167(7%). Several other studies previously reported similar sequence types among ESBL-producing strains. A study conducted in Germany identified a very high genetic similarity between ST410

E. coli strains isolated from dogs/companion animals, environment, and clinical samples. Genetic similarity of strains from different sources remark spreading potential, and further the potential threat of the clone to become a pandemic/successful clone (Schaufler *et al*., 2016). Another study from Germany among hospitalized and ambulatory patients reported a higher prevalence of strains with similar molecular pattern, ESBL-producing ST410 and ST10 *E. coli* strains (Pietsch *et al*., 2017). These two sequence types were also detected in this study, but because of a limited diagnostic capacity and infection preventive strategies in our setting, the challenge from similar sequence types and/or other local clones might continue to pose more pronounced problem.

From other studies, ST410 strains were found to harbor genes that encode carbapenemases, *bla*$_{\text{NDM-4}}$ in China (Qin *et al*., 2016), and KPC-2 from a hospital in central Greece (Mavroidi *et al*., 2012), and *bla*$_{\text{OXA-48}}$ from New Zealand among community acquired urinary tract infections (Howard et al., 2018) and also another related genetic variant of *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-181}}$ from China (Qin *et al*., 2016). It is now evident that ST410 is acquiring more resistance genes including ESBLs, pAmpC and carbapenemases. Considering the occurrence of this strain within different populations including human and animals and its ability to encode several antimicrobial resistance genes including carbapenemase; ST410 might be an evolving pandrug-resistant clone that can disseminate over a wider geographical area. Though genetic determninats of carbapenemase were not identified from ST410 in this study, the strains that belong to this clone harbor *bla*$_{\text{CTX-M-15}}$, pAmpC (*bla*$_{\text{CMY-2}}$ and *bla*$_{\text{CMY-42}}$), and several other non-ESBL-β-lactamase genes that may limit utilization of non-carbapenem alternatives and demanding use of carbapenem antimicrobials for treatment of infections caused by these strains and which in turn is a risk factor for development of carbapenem resistance.

The *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-66}}$ were the only genetic determinant of carbapenemase resistance identified. Together with both *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-66}}$, other classes of antimicrobials: *bla*$_{\text{CTX-M-15}}$, *aac(6')-Ib-cr*, *aadA2*, *bla*$_{\text{CMY-6}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM-1B}}$, *dfrA12*, *mph(A)*, *QnrS1*, *sul1*, *sul2*, and *tet(A)* were also detected. A plasmid encoded in the *bla*$_{\text{NDM-1}}$ encoding strain was also a broad-host range and able to encode multiple resistance genes, *IncA/C2*. In one previous study, *bla*$_{\text{NDM-1}}$ was identified from three *A. baumannii* strains at the same hospital (the current study site) and two of these strains were from surgical ward. And now we are reporting *bla*$_{\text{NDM-1}}$ from

E. coli in same previous same surgical ward. It might be possible that the $bla_{\text{NDM-1}}$ is spreading to other species as well.

Other multiple drug-resistant sequence types were ST648, ST10, ST405 and ST38. These strains are also epidemiologically important strains. Previously several studies reported these sequence types from environment, companion animals and humans. ST648 clone was reported multidrug resistant strain that can cause extra-intestinal infections, and also with its zoonotic potential, it might evolve as another novel international clone that may spread further (Ewers *et al.,* 2014; Muller *et al*., 2016). In an invited review that described some of these epidemiologic clones, the ability of these strains to colonize or infect both humans and animals might be the main factor contributing to the spread of the genetic determinants of antimicrobial resistance through both human and animal population (Kawamura *et al*., 2017). Another clone epidemiologically characterized in-depth and solely identified from human extra-intestinal infection is the ST131. Though ST131 is predominantly associated to hospital and community acquired infections in humans, it was also reported from water sources, food (fish and meat), and other environmental sources (Muller *et al*., 2016).

It is increasingly understood that the dissemination of ESBL-genes and specifically $bla_{\text{CTX-M-15}}$ variants is through several different sequence types. Similarly, the $bla_{\text{CTX-M-15}}$ were identified among strains characterized as belonging to several different sequence types. The most common sequence types determined were also previously reported elsewhere and some of these strains were reported to have occurred in both animals and humans as stated earlier in this document. Overall diversity of sequence types might be more than what we are currently reporting for several other strains the sequence type was not yet defined, and novel sequence types may be discovered.

Distribution and/or prevalence of sequence types that disseminate these resistance genes might vary from place to place. But there are several mechanisms that carry these strains through populations, including human to human, human to animal, animal to animal, environment, food and other related factors depending on the composition of the particular niche (Kawamura *et al*., 2017).

Rarely reported novel sequence types were identified, and similar to the common reported sequence types, these strains also encoded multiple antimicrobial resistance genes. And considering that these strains were isolated from patients seeking medical care, it might necessitates studying the novel sequence types in-depth and further vigilant tracking of these strains might uncover the spread of such strains in the setting.

Both β-lactam and non-β-lactam resistance genes are prevalent within ST410 strains. But, *bla*$_{\text{CMY-2}}$ genes were detected exclusively among ST410, whereas another but related gene *bla*$_{\text{CMY-42}}$ was detected among ST167 and ST2851. *QnrS1* genes were less prevalent, detected only among three strains, ST10, ST58 and one novel-ST (not defined). An aminoglycosides and floroquinolone resistance gene, *aac(6')-Ib-cr* is another prevalent genetic determinant of resistance among these strains. All strains of the ST44, ST167, and ST2659, followed by ST648 (93%) and ST410 (90%) strains encoded the gene. Though ST44 strains were less prevalent, all strains were identified encoding *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-1}}$, *aac(6')-Ib-cr*, *dfrA17*, *sul1/sul2*, and *catB3*.

A minimum spanning tree analysis based on seven gene MLST revealed that dissemination of resistance genes is polyclonal. ST10, ST648, ST23, ST38 were major clonal complexes, however, several other smaller clonal complexes, and singleton were observed. Furthermore, the international clone, ST131 (multi-drug resistant and a cause of extra-intestinal infections) was identified among smaller clonal complexes. Most of the prevalent STs/clones in this study were not restricted to human infections, they were also reported from previous studies conducted on strains environment, food, and animals. If these strains are left untracked both clinically and epidemiologically, it is highly likely that these strains can spread further and may cause unprecedented increase in rate of hospital aquired infections, may also continue to accumulate antimicrobial resistance genes further.

On the other hand, SNP-based phylogenetic analysis has shown nine clonally related clusters. Most of the strains in the clusters were collected from different units of the hospital. Strains in each cluster differ by 0 to ≤ 5 SNPs, and few clusters vary up to 10 SNPs within each cluster. Hence, the phylogenetic tree analysis suggests a likely outbreak along these clonal lines might have occurred in the hospital.

In conclusion, among these *E. coli* strains the rate of ESBL is very high and these strains are also resistant to several other non-β-classes of antimicrobials. A population structure revealed polyclonal clustering of strains in to different clades, including the epidemiologic clones ST410, ST10, ST648, and ST131. The multidrug-resistant and pandemic clone ST131-H30-Rx lineages also identified in this study were not previously reported from Ethiopia. The dissemination of predominant ESBL-gene the $bla_{\text{CTX-M-15}}$, and other β-lactamase genes, and non-β lactam resistance genes among these *E. coli* strains was highly likely because of a combination of horizontal genetic transfer and clonal expansion.

4.2 *K. pneumoniae* strains

Several bacterial species belonging to *Enterobacterales* are commonly isolated at clinical microbiology laboratories. Relative rate of isolation and association to a disease varies between species; however, *E. coli* and *K. pneumoniae* are predominant. *Proteus spp. Salmonella spp.,* and *Shigella spp.* are also among the most commonly reported strains associated to disease. The burden of disease causation and antimicrobial resistance varies from place to place. And also to the concordance of their abundance in nature *E. coli* and *K. pneumoniae* are the most commonly isolated strains from clinical specimen and the prevalence of ESBL strains among these clinical isolates is high (Holt *et al.*, 2015; Kawamura *et al*., 2017; Storberg, 2014).

Recently, *K. pneumoniae* has become a core issue of several studies to elucidate virulence properties/disease causation, antimicrobial resistance and alternative treatment options for serious infections (Isturiz, 2008). Not in all the cases that *K. pneumoniae* cause a disease or an infection, obviously causes a variety of opportunistic infections among immunocompromised individuals, children, old-aged people and patients with chronic illness (Adler *et al*., 2017; Moradigaravand *et al*, 2017; Gorrie *et al*., 2018).

Nevertheless, *K. pneumoniae* was also recognized as major cause of hospital acquired infections. But recently, several hypervirulent strains that can adapt to physiological conditions of apparently healthy individuals and interfere with natural defense system of humans were recognized *(*Doorduijn *et al*., 2016). These strains were also resistant to several antimicrobials commonly used for treatment of infections caused by *K. pneumoniae*: including extended spectrum cephalosporins, carbapenems, aminoglycosides, fluoroquinolones, and polymyxins. ESBL-producing *K. pneumoniae* posed a challenge for prescribers, patients and the health care in every country in the world (Holt *et al*., 2015). Moreover, high-risk clones like ST258 and its single locus variant: ST11 carbapenemase-producing *K. pneumoniae* strains called (KPC) are increasing in large parts of the world (Navon-Venezia *et al*., 2017).

The presence of large accessory genome, mobile genetic elements, and the bacterial versatility to encode virulence genes and the hypervirulence genes on chromosomal loci, together with the multidrug resistance; *K. pneumoniae* is recognized as a great threat to human health (Martin and Bachman, 2018). The increased fitness of successful clones and continuous exchange of

genetic determinants for antimicrobial resistance may birth many pan-resistant pandemic clones of *K. pneumoniae* (Cubero *et al*., 2016).

Emerging evidence from low-income countries show high prevalence of antimicrobial resistance, however, these studies are mostly phenotypic and could not answer specific and deeper epidemiological questions, and may necessitate comprehensive epidemiological studies with clear research questions. The higher burden of antimicrobial resistance on the already limited economy of LMICs is an under estimated health problem and may further compromise the health care system in these countries.

We have conducted a cross sectional study to determine the prevalence of ESBL-producing Gram-negative bacilli at Jimma Medical Center. *K. pneumoniae* was the second most prevalent species, and rate of antimicrobial resistance among these isolates was higher than all other species identified in the sudy (**Table 3.4).**

4.2.1. Prevalence: out of 146 strains collected from clinical specimens, 76% were ESBL-producing strains. All strains with confirmed ESBL-phenotypes were subjected to whole genome sequencing, and molecular features of antimicrobial resistance and population structure of these strains were analyzed. The commonly known and widely disseminated *bla*$_{\text{CTX-M-15}}$ was the most prevalent ESBL-gene detected among 84% of ESBL-producing strains. The strains encoded also for several other ESBL-types, non-ESBL-β-lactam resistance genes, and non-β-lactam antimicrobial resistance genes. Most of these strains were resistant to at least three classes of antimicrobials. The prevalence of *bla*$_{\text{CTX-M-15}}$-producing strains is very high as compared to recent studies reported from different parts of the world.

A study from Mali, Bamako, reported very high prevalence of ESBL, 58.5% among overall *Enterobacteriaceae*, 76.9% among *K. pneumoniae* strains, and 62.3% among bacteremic patients (Sangare *et al*., 2017). In a study from Mali the predominant ESBL-type was *bla*$_{\text{CTX-M-1}}$ as compared to the *bla*$_{\text{CTX-M-15}}$ in the current study. On the other hand, there were studies that reported a high prevalence of ESBLs in *E.coli* and *K. pneumoniae* fromTanzania, South Africa, Gabon, Guinea Bissau, and many other parts of the continent and the predominant type of ESBL was *bla*$_{\text{CTX-M-15}}$ (Saravanan *et al*., 2018; Storberg, 2014).

Most of the studies conducted in sub-Saharan African countries were either only phenotypic and/or small sample size molecular studies. The problem might have been under estimated in the absence of standard techniques and lack of appropriate expertise to detect and confirm ESBL-phenotypes/genotypes. Also, the small sample size molecular studies might not reveal the accurate picture. A recent review on antimicrobial resistance in Africa, reported lack of updated data for nearly 40% African countries and the review also noted that quality of microbiological data is a serious concern for those data is available (Tadesse *et al*., 2017).

Our study being conducted unarguably on large sample size, molecular typing performed with whole genome sequencing at a highly satandardized laboratory (one of Europe's centers of excellence), we report a reliable evidence of surging antimicrobial resistance in a rural setting of Africa. Besides, all other emerging reports from Africa also showed the prevalence of ESBL particularly *bla*$_{\text{CTX-M-15}}$ mediated resistance is increasing among *K. pneumoniae* and other *Enterobacteriaceae* strains.

On top of resistance to extended spectrum cephalosporins most of these strains were resistant to other important classes of antimicrobials used for treatment of infections caused by *K. pneumoniae*. A total of 22.6% of these strains were non-susceptible to carbapenems, however, only one of these strains detected encoding for *bla*$_{\text{NDM-1}}$ genes. Otherwise, no carbapenemase gene was detected. Carbapenemase-producing *K. pneumoniae* were prevalent in different parts of the world and when detected from clinical specimens as a cause of serious infections; it is challenging to treat. Nevertheless, the prevalence of carbapenemase mediated carbapenem resistance is low in Africa in general. The low prevalence of carbapenem resistance from Africa might be mainly because of limited availability of carbapenems in clinical use, and/or partly because of the lack of comprehensive studies that can assess the actual burden of antimicrobial resistance in-depth (Tadesse *et al*., 2017).

A higher burden of carbapenem resistance has relatively been reported from other parts of the world, 37% in a continental survey from Europe (Grundmann *et al.*, 2017), 25% in a network surveillance of acute care hospitals from USA (Han *et al*., 2017), and yet another systematic review reported a national incidence of CRE as low as 0.3-2.9 per 100,000 patient-years (Livorsi *et al*., 2018), in Asian countries (57% in 2014 in India (Kakkar *et al*., 2017), and 14% in 2015 in China (Hu *et al*., 2016) was reported. The Indian sub-continent is a hot spot for

bla$_{NDM-1}$ resistance genes as an initial source of dissemination. The prevalence of carbapenem resistance is therefore significantly higher than the current report from Ethiopia. Though the prevalence is low, carbapenem resistance was comparatively better reported from South American countries compared to Africa. A single case of *bla*$_{NDM-1}$ producing *K. pneumoniae* was reported from Ecuador in 2017 (Romero-Alvarez *et al*., 2017). Evidences of resistance genes of carbapenem resistance including KPCs were reported from Colombia, Venezuela, Brazil, Mexico, and Argentina (Lee et al., 2016; Logan and Weinstein, 2017). Recently strains with multiple carbapenemase genes were detected in Vietnam including: KPC-2, *bla*$_{NDM-1,}$ *bla*$_{NDM-4}$ and *bla*$_{OXA-48}$ (Tada *et al*., 2017).

Higher prevalence of resistance to non-β-lactam antimicrobials was also observed among *bla*$_{CTX-M-15}$ producing strains, thus compromising susceptiblity of these strains to alternative antimicrobials recommended for treatment of ESBL-producing *K. pneumoniae*.

Analysis of the antibiogram (**Table:3.17**), shows that 93% of these strains were resistant to at least three classes of antimicrobials, and 75% of the strains were resistant to at least four class of antimicrobials. Moreover, 15% of strains were non-susceptible for nine different drugs that belong to six classes of antimicrobials revealing the potential for pan-drug resistance. A similar magnitude of multiple drug resistance has been reported from Kenyan rural hospital among invasive infections caused by *K. pneumoniae* strains (Poirel *et al*., 2011). Few other studies, also from Kenya reported low prevalence of *bla*$_{NDM-1}$ carbapenem resistance among *K. pneumoniae* at Nairobi tertiary hospital, and these *K. pneumoniae* strains were also resistant to several other classes of antimicrobials (Poirel *et al*., 2011; Revathi *et al*., 2013).

4.2.2. Molecular features of antimicrobial resistance: three types of class-A extended spectrum β-lactamase encoding genes were detected. These genes were commonly identified genetic determinants, *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$. The *bla*$_{CTX-M}$ was the most prevalent where *bla*$_{CTX-M-15}$ (84%) was the predominant variant of the *bla*$_{CTX-M}$ genes, followed by *bla*$_{CTX-M-11}$(3.6%). Only few strains that encode for other variants (*bla*$_{CTX-M-14}$, *bla*$_{CTX-M-3}$ and *bla*$_{CTX-M-9}$) were observed. High prevalence of ESBLs and other non-ESBL β-lactamases were detected among these *K. pneumoniae*. Previous studies from Kenya and other East and Central African countries reported a higher prevalence of *bla*$_{CTX-M-15}$ ESBLs, and now it is apparent that *bla*$_{CTX-}$

$_{M-15}$ is disseminating in East Africa countries: Ethiopia, Kenya, Tanzania and Uganda (Henson *et al*., 2017; Moses *et al*., 2014; Sonda *et al*., 2018).

Multiple β–lactamase resistanc genes, *bla*$_{SHV-12}$, *bla*$_{SHV-26}$, *bla*$_{SHV-27}$, *bla*$_{SHV-28}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, and *bla*$_{OXA-10}$ were observed among these *K. pneumoniae* strains. From the many β-lactamase genes and genes to other classes of antimicrobials among *K. pneumoniae*, the *bla*$_{CTX-M-15}$ was most prevalent . Though, multiple molecular types were detected *bla*$_{CTX-M-15}$ is singled out as the main mechanism of resistance to extended spectrum β-lactamase in the setting.

Global differences in the proportion and distribution of *bla*$_{CTX-M}$ variants is evident, but currently *bla*$_{CTX-M-15}$ is the most commonly identified ESBL-gene from different sources and might have contributed to a larger proportion of global burden of antimicrobial resistance. On the other hand, except the one *bla*$_{NDM-1}$ strains that also harboured *bla*$_{CMY-6}$, pAmpC genes were not detected among these strains. The rate of *bla*$_{CMY}$ genes is lower, but because the gene was carried on genome of high-risk MDR strains (probably on the broad-host-range *IncA/C* plasmid detected in the same strain), and together with the presence of non-β-lactam resistance genes the probability of selection by random prescription is higher, and hence, it might surge in a very short time. A comparative plasmid genome study done by Carattoli *et al,* envisioned *bla*$_{CMY-2}$ encoding broad-host-range *IncA/C* plasmid scaffold might support the large scale diffusion of *bla*$_{NDM-1}$ through Gram-negative bacilli (Carattoli *et al*., 2012). Previous study from same study site reported three starins *A. baumanii* with *bla*$_{NDM-1}$ gene (Pritsch *et al*., 2017). The *bla*$_{NDM-1}$ was reported in different species of Gram-negative bacilli from African countries. It is emerging evidenced also in other east African countries like Kenya, Uganda and Madagascar (Carattoli *et al*., 2012; Holman *et al*., 2017; Poirel *et al*., 2011; Pritsch *et al*., 2017). Even though the prevalence of carbapenem resistance is relatively low, it must be carefully tracked/monitored.

We are not aware of any official documentation of the use of carbapenems in our setting for the last decade. Infection prevention strategies are poorly instituted, alternative treatment options are rarely available or recognized. On top of the limited economy, and high disease burden in the region, occurrence of any level of carbapenem resistance together with higher prevalence of ESBLs is an early warning of a looming antimicrobial resistance related health care problems.

4.2.3. Resistance to non-β lactam antimicrobials: resistance to aminoglycosides, one of the most important antimicrobials for treatment of infections caused by Gram-negative-bacilli, is another challenge in the field of medical care and public health. Several genetic determinants of aminoglycosides resistance commonly known as: *aac, aad, aph*, and *str*, were detected in *K. pneumoniae* strains. From the resistance genes encoding for aminoglycosides, the *aac(6')-Ib-cr* (65.1%) is the most prevalent, and 95.7% of the *aac(6')-Ib-cr* strains are $bla_{\text{CTX-M-15}}$ producing. Though the 4.3% of *aac(6')-Ib-cr* do not encode the $bla_{\text{CTX-M-15}}$, they encode other ESBL-types either other $bla_{\text{CTX-M}}$ types or bla_{SHV} types. The co-existence of $bla_{\text{CTX-M-15}}$ and *aac(6')-Ib-cr* in the same strain was statistically significant (P-value < 0.0001). A potent mechanism of aminoglycoside resistance, the plasmid-mediated 16S rRNA methylase (*rmt*) genes were not detected.

The co-existence of ESBLs and resistance genes encoding resistance to both aminoglycosides and fluoroquinolones *(aac(6')-Ib-cr*)) may significantly limit the use of first-line and second-line antimicrobial options for treatment of infections caused by resistant strains. The situation might be worse for serious infections caused by strains with multiple resistance gene profiles. Moreover, when it happens in low-income countries, where diagnostic capacity is limited and alternative treatment options/antimicrobial are not available, the problem with antimicrobial resistance can cause the most of damage.

A study from Tehran reported a high prevalence of aminoglycoside resistance among $bla_{\text{CTX-M-15}}$-producing *K. pneumoniae* strains. But the prevalence of *aac(6')Ib-cr* among $bla_{\text{CTX-M-15}}$ strains in this study is much higher than the study from Tehran (Peerayeh *et al*., 2014). Also, a study from Egypt revealed that *aac(6')-Ib-cr* was prevalent among *K. pneumoniae* isolates (El-Badawy *et al*., 2017). A more comprehensive genomic analysis study conducted on complete and draft genomes at NCBI databases revealed that *aac(6')-Ib* and *aac(6')-Ib-cr* genes are more common in *E. coli* and *K. pneumoniae* than other species, but the rate varies between regions and mor specifically institutions. The aac-variants of aminoglycosides resistance genes were widely identified among strains from human, environment, and animals. But the "**cr**" variants were more common in animals and environment than humans (Kim *et al*., 2018). In light of this, species from animal origin yet able to colonize human are the reservoir of these variants of genetic determinant for aminoglycoside and floroquinolone resistance. And hence,

it might be possible to assume that strains in this study with *aac(6')-Ib-cr* and *bla*$_{\text{CTX-M-15}}$ can have more dimension of spread between environment, animals and human population.

Investigation of fluoroquinolone resistance genes revealed presence of *QnrS*(16.5%), *QnrB6* (11.9%), and *qepA* (0.9%). Qnr-enzymes mediated quinolone resistance is low. Furthermore, genetic determinants of resistance to other classes of antimicrobials that includes: phenicols, rifampicin, trimethoprim, tetracycline, sulfonamides, macrolides, and fosfomycin were also determined. We identified that genetic determinants of resitance to these drugs exist in various proportions and combinations. This is yet another evidence of multiple-drug resistance, and might also indicate a non-selective use of antimicrobials in the setting.

K. pneumoniae is recognized as both major source and shuttle of antibiotic resistance, and it is increasingly reported from hospital and community acquired infections (Navon-Venezia *et al.*, 2017). Several studies evidenced increased prevalence of resistatnce among *K. pneumoniae* strains isolated from food, water surfaces, environment, and animals (wild and companion) (Kohlenberg *et al*., 2012; Liebana *et al*., 2013; Messai *et al*., 2008; Podschun and Ullmann, 1998).

The implication of detecting multiple factors of antimicrobial resistance in a single strain is beyond presenting only increased prevalence of an antimicrobial agent. A single strain that has developed resistance to several of antimicrobials currently in use should concern prescribers, and public health authorities (Holt *et al.*, 2015). Such strains should be identified as early as possible and treated with stringent public health and infection control measures. Otherwise, as these strains persist in their niche, it is highly likely that these strains and/or the factors of resistance (plasmids, transposons, and insertion sequences) may disseminate either vertically or horizontally from same species/strains through to other genuses and may populate to a wider context of challenging epidemiology.

Therapeutic options for infections caused by ESBL-producing strain that also encode other non-ESBL-β-lactamases might be challenging to treat serious infections. The carbapenem-sparing strategies including use of piperacillin-tazobactam, cefepime, and β-lactam/β-lactam inhibitor combinations can be hampered by several specific reasons including severity of infections, bacterial load, dosing, and scheduling. Thus, necessitating the use of carbapenems,

which is again risk factor for development of carbapenemase-mediated carbapenem resistance, and so doing to colistin, the last resort antimicrobials.

A very high prevalence of ESBL and co-existence/resistance of genetic determinants of multiple classes of antimicrobials among *K. pneumoniae* strains calls for an urgent intervention from multiple fronts. Cognizant of the limited economy the country invested in health care, the solution demands novel, cost effective and efficient strategies to control these multi-drug resistant clinical isolates.

4.2.4. Plasmids: plasmids are regarded as mainly responsible for the horizontal transmission of ESBL-genes, and most importantly responsible for worldwide dissemination of the $bla_{\text{CTX-M-15}}$. *In silico* based replicon typing identified nine different types of plasmids. These plasmids were the most common plasmids identified in *Enterobacterales. IncFIB(K/Mar/pQil)* (32.9%) is most prevalent followed by *IncR* (24.7%), *IncFII* (14.4%) and *IncQ1* (12.4%). *IncF* family is most prevalent family of plasmids (identified among 48.03% of *K. pneumoniae* strains). From *IncF* family, the *IncFIB*, *IncFII* and *IncFIA* are usually regarded as epidemic plasmids. Because of their presence in a number of species of bacteria of different origin and also reported from different countries, plasmids can be used as an important epidemiological tools (Carattoli, 2009). All plasmids identified in the study except *IncQ1* and *ColE* were conjugative plasmids that can spread between several genera. The conjugative plasmids are usually large (>50kb) and mostly carry multiple genetic determinants conferring resistance to different classes of antibiotics (Carattoli, 2013).

In most cases, plasmids that encode ESBLs and fluoroquinolone resistance were conjugative plasmids (Carattoli, 2009; Carattoli *et al*., 2012; Navon-Venezia *et al*., 2017). The conjugative plasmids can encode also for virulence factors and other factors related to the maintenance and stability of the plasmid itself, such as toxin-antitoxin addiction systems (Schaufler *et al*., 2013). In this study, the *IncF* plasmids were detected from fifteen different sequence types- ST218, ST17, ST15, ST147, ST45, ST20, and ST340. *IncF* replicons being the most prevalent and/or successful plasmids, the emergence and spread of resistance to multiple antimicrobial classes at this study area might be because of *IncF* plasmids. *IncF* plasmids can also carry virulence genes and because strains bearing these plasmids can endure antimicrobial selection pressures,

and also possibly contributed for the selection and propagation of resistant and virulent strains within a population of bacteria (Shintani *et al*., 2015).

The *IncQ1* plasmids were also detected among epidemic clones. However, the role of *IncQ1* plasmids in dissemination of resistance genes might not be as efficient as conjugative plasmids. *IncQ1* plasmids are not conjugative plasmids, but highly promiscuous/mobilizable replicons and can replicate in a strand displacement replication mechanism in a variety of bacterial hosts. The presence of highly mobilizable *IncQ1* in an epidemic clone together with a helper plasmid at the same time, might be considered as a high-risk of MDR outbreak. An international clone ST45, identified in this study carry the conjugative plasmid *IncFIB* and the highly mobilizable *IncQ1*. A previous study from Portugal reported KPC-3 producing and mcr-1 positive ST45 strain as a cause of an outbreak in a hospital (Mendes *et al.*, 2018). Hence, ST45 is an epidemic clone and capable of encoding multiplel plasmids. As also stated elsewhere in this document another ST45 was reported encoding for *IncA/C2* plasmid. Thus, it might be possible that this epidemic clone may spread further. *IncR* plasmids were also identified from epidemic clones: ST15 and ST147. Overall, most of plasmids identified in this study are broad-host-range plasmids capable of carrying and transferring MDR and virulence genes. Only three of the plasmids identified (*IncR*, *IncQ1*, and *Col*) were mobilizable plasmids (Rozwandowicz *et al*., 2018).

The type of plasmids disseminating in bacterial population can influence diversity of resistance profiles. Studies from Nigeria (Ogbolu *et al*., 2013) and Ghana (Agyekum *et al*., 2016) and Central Africa Republic (Rafaï *et al*., 2015) reported that *IncF* plasmids were predominant and might be responsible for increased prevalence and dissemination of several different β-lactamase genes: bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$ in Gram-negative-bacilli (mainly among *E. coli* and *K. pneumoniae*). In European countries like Bulgaria (Markovska *et al*., 2014), Greece (Peirano *et al*., 2014) and Portugal (Vubil *et al*., 2017) where the prevalence of AMR is high, a concordant predominance of *IncF* plasmids were observed. Such evidences may indicate that targeting replicon typing plasmids might be an important area of epidemiology. But the concept requires further in-depth studies that may provide novel ideas on early detection, and tracking of antimicrobial resistance. A study with a novel approach using optical DNA mapping of plasmid genomes have showed an outbrek in a neonatal ward (Müller *et al.*, 2016).

The high prevalence and diversity of plasmids among *bla*$_{CTX\text{-}M\text{-}15}$-producing strains and presence of multiple resistance determinant genes for multiple classes of antimicrobials among several epidemic clones indicates a high-risk of treatment failure and an on-going evolution of XDR and/or PDR. Yet, the evolution of XDR and PDR strains depend on the presence of large conjugative plasmids that can integrate many resistance determinant genes (multiple physically linked resistance genes) on one plasmid. Further selection of these strains for the antimicrobial for which they are now susceptible carry a high-risk particularly in serious infections/invasive infections that may demand alternative treatment options. In many situations, an unavoidable empirical treatment itself might have contributed for selection of more resistant genes for already MDR strains, for instance, carbapenemase production.

4.2.5. Virulence factors of *K. pneumoniae* strains: strains of *K. pneumoniae* were primarily recognized as opportunistic pathogens that may cause opportunistic infections. But there are *K. pneumoniae* strains that can also cause community acquired infections including bacteremia/sepsis, pneumonia, UTI, meningitis, wound infections, and pyogenic liver abscess. Several of the clinical strains were reported from infections associated to use of indwelling-devices and also compromised immune response. The classical *K. pneumoniae* strains were known for causing serious infection among immunocompromised individuals, but recently, the emergence of hypervirulent strains increased the susceptibility of apparently healthy and/or immunocompetent individuals. The co-evolution of hypervirulence and multi-drug resistance in *K. pneumoniae* may be challenging. Furthermore**,** *K. pneumoniae* may employ several strategies to protect themselves from host immune response. In fact, there might be significant heterogeneity among these strains, not every virulence factor plays the same role in all of them. The role of each virulence factor depends on tissue specific responses, however, most proteins function in metabolism and regulation of transcription. The main virulence factors widely described include: capsule, lipopolysaccharides, siderophores, and fimbriae. The virulence genes identified among strains in this study were grouped into four categories: mainly protectins (capsule and/or lipopolysaccharides), siderophores/iron scavenging molecules (enterobactin, yersinobactin, salmochelin, and aerobactin), fimbriae (Fim-1, and Fim-3), and also allantoin metabolism proteins (*allA*, *allB*, *allC*, *allD*, *allR*, *allS*) (**Table 3.20**).

Capsular typing was done based on *cps* genes/K-loci typing using *Kaptive*, (a web-tool for capsular and polysaccharide typing of *K. pneumoniae*). Forty different capsular types were detected, the most frequently identified capsular types were KL62 (9.26%) and KL57 (9.26%), followed by KL64 (6.48%) and KL25 (6.48%). Though, K1 and K2 are commonly reported from virulent strains, only K2 (1.85%) was identified among these strains. However, the most prevalent capsular type KL57 is a feature of hypervirulent strain. Several studies previously reported KL57 strain is hypermucoviscous (HV), and strains encoding the capsular type are considered one of the virulent strains of *K. pneumoniae*. All the KL57 strains were identified with both *rmpA* and *rmpA2* genes exclusively among an epidemic clones of ST218. Only a single strain of ST198 group and KL30 capsular type *K. pneumoniae* also harbor both *rmpA* and *rmpA2* genes. The presence of KL57 capsular locus with *rmpA/rmpA2* genes in the genome of ST218 strains was statistically significant (Fisher exact test, (p-value < 0.0001)).

A comprehensive study from Russia, on genomic analysis of the virulence genes and animal experiment to anlyze virulence properties charcterized the ST218-KL57 *K. pneumoniae* strains as hypermucoviscous and consequently hypervirulent strains (Lev *et al*., 2018). Moreover, these strains also encode several other virulence genes, siderophores (*iroB*, *iroC*, *iroD*, *iroN*, *iucA*, *iucB*, *iucC*, *iucD*, *iutA*, and yersinobactin complex genes (*ybtA*)), and FimH-1 and *mrkABCD* gene cluster (*FimH-3*). According to some epidemiological evidences from China and Russia, KL57 capsular types are associated with diarrhea, pyogenic liver abscess, meningitis, and blood stream infections (Liao *et al.*, 2014; Zhang *et al*., 2018). Another study from China, reported KL57 strain with multiple other virulence factors and carbapenem resistance among ST163 *K. pneumoniae* strains (Qiong *et al.*, 2018).

Although they encode multiple resistance genes for several classes of antimicrobials, KL57-ST218 strains in this study were primarily selected for ESBL phenotype. Furthermore, these strains are distinct on the pattern of O-Lipopolysaccharide surface molecules as well, all of them identified as O2v2 O-LPS. The presence of similar pattern of surface molecules (KL-locus and O-Lipopolysaccharides) on the surface of high-risk and hypervirulent clones of *K. pneumoniae* is an important information for intervention strategies targeting such surface molecules expressed on an epidemic clones. In this era, the prevalence of resistant strains and level of resistance is increasing and there is palpable lack of enough alternative antimicrobials

for treatment. Vaccination-based prevention and control of superbugs is possibly one strategy and for development of vaccine, the knowledge of such a pattern and combination of surface molecules and/serotype is important. We present here, a scenario of unique pattern of O-LPS and capsular serotypes on the surface of hypervirulent and multidrug resistant strain. And yet an epidemic clone, which may became a global health concern and harbor other multiple virulence factors. However, more similar evidences might be vital for vaccine development and vaccination-oriented prevention strategies.

Prevalence of KL2 capsular type strains is lower. Other, virulence related capsular types rarely identified include KL20 and KL54. Most importantly the antimicrobial resistance genes are spreading thorough strains with diversified capsular serotypes (KL-locus).

Another protectin studied was the O-lipopolysaccharide used for colonization/attachment to host cells, and helps for interaction with immune cells and/or immune molecules including complement (Evrard *et al*., 2010). O-lipopolysaccharide surface molecules of *K. pneumoniae* are commonly classified nine types (O1 to O9). But an increased availability of whole-genome data and bioinformatics tools enhanced resolution of the scanning capacity of scientists through bacterial genome revealed novel variants of lipopolysaccharides. We used the whole genome based serotype prediction tool called ***Kaptive*** (Wick *et al.*, 2018). A total of 12 variants of O-lipopolysaccharide molecules were identified and the most prevalent variants were O1v1 (31.48%), O1v2 (21.22%), O2v2 (12.96%), O2v1 (10.19%) and O4 (8.33%). Nevertheless, main O-LPS were O1 (53.66%) and O2 (23.15%). Epidemiological studies concerning the prevalence and distribution of O-LPS in different disease conditions were scarce. According to few previous studies the O1-LPS has a role in causation of pyogenic liver abscess in K2 capsular type strains (Hsieh *et al*., 2012) and more generally O-LPSs were studied as an important potent immunogen that can modulate an immune response and O-LPS loss can also alter the protein structure and interaction of the bacteria to its surrounding (Cahill *et al*., 2015; Doorduijn *et al*., 2016; Paczosa and Mecsas, 2016). Despite a resurgent need of a deeper understanding of capsular and lipopolysaccharide surface molecules, which can be a potential vaccine candidate in the era of genomics and a relative availability of whole genome data, much of the work is yet to be done.

On the other hand, all *K. pneumoniae* strains encoded the chromosomal type-1 (*fimA*, *fimB, fimC*, *fimD*, *fimH, fimI*) and type-3 (*mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkF*, *mrkI*, *mrkH*, *mrkJ*) fimbrial gene cluster..

Genetic determinants of siderophores/iron chelating molecules were one of the virulence genes relatively well studied (Paczosa and Mecsas, 2016). The highest prevalence of siderophore in this collection was the yersinobactin (*ybtQ*) (42.5%), followed by aerobactin (*iutA*) gene cluster (9.2%), and salmochelin (*iroN* gene*)* cluster (7.4%). Higher prevalence of siderophore genes were observed among strains that encode: KL57, O2v2, and *rmpA*/*rmpA2* genes. All strains that belong to ST218 that possess KL57 capsule and O2v2 O-LPS serotypes demonstrated highest prevalence of salmochelin (*iroN*) and yesrsinabactin (*ybtQ*). Moreover, all these strains encode for the aerobactin gene cluster (iutA). With all sort of genetic determinants we have studied, this cluster is a multiple drug resistant and hypervirulent by demonstrating not only hypermucoviscous but also expressing several types of strong iron chelating molecules, which is vital for colonization, and progression of the bacterium in healthy/immunocompetent host cells.

Though, it has been a tradition to describe *K. pneumoniae* as opportunistic pathogen, studies reporting multiple virulence factors from MDR strains of *K. pneumoniae* that belong to different clones were increasingly reported from different parts of the world (Turton *et al*., 2018). Strains identified with presence of multiple virulence factors, not only capsule and *rmpA* genes but also other factors (siderophores and distinct O-lipopolysaccharides) were associated to serious infections including pneumonia, blood stream infections and pyogenic liver abscess (Cubero *et al*., 2016; Hsieh *et al*., 2012; Liao *et al*., 2014; Turton *et al*., 2018). There were reports of serious infection from classical strains *K. pneumoniae* without hypermucoviscous genetic determinants including *rmpA*, *magA*, and the K1 and K2 capsular types, which usually are used to describe strains as hypervirulent.

In the era of whole genome sequencing and genomics both species and strain level evidences are being generated and accumulated. Knowledge on genomic evolution, horizontal genetic transfer, and mobile genetic elements specifically about plasmids drastically increased. Several studies showed that virulence and antimicrobial resistance factors are being continually

exchanged between species and often genera. It is time to reconsider the consensus definitions of virulence and hypervirulence for both susceptible and resistant strains of *K. pneumoniae*.

Other miscellaneous genetic factors that might be considered as virulence and/or resistance determinant factors were also analyzed. These factors include: *allS* (allantoin metabolism gene cluster), outer membrane proteins (*OmpA*, *OmpK35* and *OmpK36*), *AcrAB* (efflux pump for virulence and resistance factors) and ABC transport systems (*kfuA*, *kfuB*, *kfuC*). *OmpK35* and *OmpK36* are an important porin channels for influx of large antibiotic molecules including cephalosporins and cefepime. A mutational loss of these porin proteins decreases the rate of influx of antimicrobials across cell membrane and hinder entrance of antimicrobials and consequently the drug may not get to the target site (Sugawara *et al.*, 2016). The phenomenon gives bacteria the ability to grow in microenvironment that contain previously appropriate dose of antimicrobial to prevent growth of these bacteria.

A recent study from USA revealed porin deficiency was responsible for reduced susceptibility to ertapenem among strains lacking carbapenemases (Wise *et al*., 2018). Another study described a phenomenon of strains non-susceptible to carbapenem and lacking carbapenemase, but altered and/or deficient *OmpK35*/*OmpK36* caused an outbreak in hospitals (Poulou *et al*., 2013). Such building evidences/scientific reports quest for more exploratory works on the membrane channels or diffusion porins of outer membrane proteins. Understanding the role of membrane proteins both influx and efflux pumps can increase in the pool of knowledge about mechanism of antimicrobial resistance and effort to combat super bugs (Ishan and Shanshak, 2018; Sugawara *et al*., 2016; Wise *et al.*, 2018).

4.2.6. MLST and Epidemiologic strains: a multi-locus sequence typing (MLST) scheme and SNP-based phylogetic study were performed for these *K. pneumoniae* strains. These strains were categorized into 42 different sequence types. Most commonly identified sequence types were ST218, ST15, ST147, ST17 and ST39. ST218, ST17 and ST15. Most of the sequence types were also reported from previous studies as strains harboring virulence factors and resistance genes. As discussed elsewhere in this document, study from Russia reported that ST218 *K. pneumoniae* isolates were associated with antimicrobial resistance and virulence compared other sequence types (Lev *et al*., 2018). Similarly, a study from UK described ST147

and ST15 encoding both virulence genes, and resistance genes (ESBLs) (Turton *et al*., 2018). The other sequence types were also associated to multiple genetic determinants of antimicrobials, both β-lactam and non-β-lactam agents. Studies from both UK and Russia revealed multiple resistance genes to different classes of antimicrobials that includes quinolones and trimethoprim (Lev *et al*., 2018; Turton *et al*., 2018). According to a another study from Korea, *bla*$_{\text{CTX-M-15}}$ genes were encoded by polyclonal strains, and most prevalent strains similar to sequence types identified in this study. These include: ST218 (CC163), ST15 (CC14), ST17 (CC17), and ST39 (CC39) (Ko *et al*., 2010)..

Epidemic/successful clones described in several previous studies were detected in this setting. These clones encode *bla*$_{\text{CTX-M-15}}$, *acc(6')-Ib-cr*, *bla*$_{\text{SHV}}$-genes (*bla*$_{\text{SHV-11}}$, *bla*$_{\text{SHV-28}}$, *bla*$_{\text{SHV-26}}$) and other resistance genes including: ST15, ST17, ST14, ST20, ST147, ST340, and ST10. In previous studies elsewhere, many of these clones were reported from invasive and non-invasive infections (Ko *et al*., 2010; Nielsen *et al*., 2011; Turton *et al*., 2018). The fact that these strains can persist in clinical settings may cause further antimicrobial selection, and their ability to carry virulence factors that may enable them to evolve to multiple resistant strains able to cause untreatable invasive infections (Paczosa and Mecsas, 2016).

ST15 *K. pneumoniae* encoding both *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{SHV-28}}$ is second prevalent clone in this study. Similar epidemic clone was previously reported from Copenhagen (Nielsen *et al*., 2011). In similar manner, ST101 that encoded the *bla*$_{\text{CTX-M-15}}$ also identified in this study has been previously reported from Hungarian teaching hospital together with the epidemic clones ST15 and ST147 (Melegh *et al*., 2015).

The pandemic carbapenemase clones ST23, ST11 and ST258 (Navon-Venezia *et al*., 2017; Shi *et al*., 2018) were not identified. ST11 was reported as KPC variant common in China, and ST258 are mostly reported from US and Europe (Dong *et al*., 2018; Ko *et al*., 2010; Tängdén and Giske, 2015). In African countries the prevalent carbapenemase so far is the *bla*$_{\text{NDM-1}}$. The dissemination of the *bla*$_{\text{NDM-1}}$ is through several novel sequence types and different species of the order *Enterobacterales* (Carattoli et al., 2012; Henson et al., 2017; Poirel et al., 2011; Pritsch et al., 2017).

Despite the lower prevalence of carbapenemase at this study, the strain encoding the *bla*$_{\text{NDM-1}}$ gene, ST45 is an epidemic clone. In a study from Portugal, ST45 *K. pneumoniae* (which is KPC-3 and *mcr-1* positive) caused an outbreak in a hospital was reported (Mendes *et al*., 2018). None of the studies so far reported *bla*$_{\text{NDM-1}}$ from ST45. This study is the first to report *bla*$_{\text{NDM-1}}$ from ST45 *K. pneumoniae*. We believe this strain is a high-risk clone that may continue to spread locally unless appropriate intervention is instituted. This strain possess a broad-host plasmid *IncA/C2*, and because *bla*$_{\text{NDM-1}}$ carrying *IncA/C* plasmids previously reported from *K. pneumoniae* strains were able to capture other resistance genes too (Carattoli *et al*., 2012); it is likely that these carbapenemase gene (*bla*$_{\text{NDM-1}}$) can easily disseminate to other strains and through to other species.

Moreover, the dissemination of resistance genes, mainly therough *bla*$_{\text{CTX-M-15}}$ seem to be disseminating along several clonal lines. In an SNP-based phylogenetic analysis, several strains isolated from different units of the hospital were clustered into a cluster of related strains with an SNP differences of zero to maximum of ≤10 SNPs between strains in a given cluster. The overall dissemination of resistance genes seems to be through both vertical and horizontal genetic transfer, that involves local and international clones together with epidemic plasmid capable of carrying and transferring MDR genes. These multiple clusters might have been an independent outbreak of *bla*$_{\text{CTX-M-15}}$ strains through multiple clonal lines

In general, most of the *K. pneumoniae* strains detected are high-risk clones. The dissemination of the multiple-drug resistance including ESBLs is polyclonal. It includes epidemic clones, singletons, common clonal complexes, and novel strains). Moreover, the rate of combination resistance to several classes of antimicrobials is high, and unrestricted use of antimicrobials may allow to continuously accumulate antimicrobial resistance genes for some strains to evolve to XDR and/or PDR. An immediate concern is that these strains are mostly resistant to antimicrobials available and routinely prescribed at the study site. Because these clones are high-risk clones, continuous monitoring and controlling should be a priority.

Possible risk factors for acquiring ESBL-producing *Enterobacteriaceae*

Enterobacteriaceae are common residents of the clinical environmentband hence admission to the hospital has been reported to be a risk factor in several studies including the findingsnof this data. Other risk factors identified in this study include, age lessthan 5 years, presence of underlying chronic illness, admission to surgical ward.

A very high prevalence of ESBL-producing *Enterobacteriaceae* at a tertiary care hospital serving a hugely populated regions of the country as both referral and teaching hospital is an important epidemiological data. Previous studies at the hospital have shown the antimicrobial usage is sub-standard, miss use and over use were reported. Strict prescription policy, strong and reliable infection control strategies and Antimicrobial stwewardship are not seem to have been functional.

On top of the limited economy of the country and lack of sufficient antimicrobial agents, the higher prevalence of irrational use of these drugs may further fuel the current problem. Lack of standardized clinical microbiology laboartory and the pool of experts to guide clinicains prescribing the antibiotics with as much as possible with depth of information about the strains prescribing might be extremely challenging.

If these problem can not be tackeled as soon as possible, the vulnerable segment of the population, like neonates, elderly, immunocompromised patients, malignancy, diabetic and people with other chronic illnesses will be left to a greater health risk. Furthermore, the presence of multidrug resistant and invasive/hypervirulent starins together with some of them encoding carbapenemase genes at this setting shows greater challenge lying ahead for the general public.

CHAPTER 5: CONCLUSION

The prevalence of ESBL-producing *Enterobacteriaceae* strains at this hospital is very high. These strains were isolated from different units of the hospital and from patients of all ages. Most commonly isolated and widely distributed strains *E. coli* and *K. pneumoniae* were the main drivers, and these strains were also resistant to other non-β-lactam antimicrobials. These *E. coli* and *K. pneumoniae* strains carry multiple genetic determinants of resistance genes and virulence.

The $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{OXA-1}}$ were the most commonly identified genetic determinants of β-lactamases. $bla_{\text{CTX-M-15}}$ was the predominant ESBL gene identified in both *E. coli* and *K. pneumoniae*. Several bla_{SHV}-subtypes were prevalent among *K. pneumoniae* strains and rarely identified in *E. coli* strains. High prevalence of resistance to aminoglycosides, floroquinolone, trimethoprim, sulfonamides and other antimicrobials were observed in both *E. coli* and *K. pneumoniae* strains. Nevertheless, most of these strains were susceptible to carbapenems. On the other hand, *IncFIB* replicons (conjugative plasmids) that can carry MDR and virulence genes, and can facilitate their own transfer were prevalent plasmids at the study area.

Dissemination of ESBL genes, mainly the $bla_{\text{CTX-M-15}}$, was through polyclonal lines of bacterial strains in both *E. coli* and *K. pneumoniae*. Several of the clonal clusters were international clones. A phylogenetic analysis of several clusters of strains demonstrated that these strains were closely related strains and a it is highly likely that the strains were part of an outbreak. An international clone of *E. coli* strain ST131-H30-Rx was possibly evolving unrecognized in the hospital. Similarly, an international clone and hypervirulent strain of *K. pneumoniae* ST218-KL57-O2V2 was prevalent among *K. pneumoniae*.

In summary, we report very high prevalence of multidrug-resistant, $bla_{\text{CTX-M-15}}$-ESBLs. The resistant strains were polyclonal and resistance genes were disseminating along several clonal lines. Most of these clones are epidemic clones reported elsewhere. The very high prevalence of ESBLs, presence of epidemic plasmids with epidemic clones/strains in the absence of sufficient antimicrobial alternatives in a country with high population burden and limited economy allocated for antimicrobial resistance, the burden of antimicrobial resistance may

surge to a level that might be beyond control. And hence, this study calls for an urgent response to tackle the problem.

Recommendation

We recommend expanding and strengthening capacity of microbiology laboratory at its best possible capacity. It is unarguably important to rely on microbiological laboratory evidence to prescribe antimicrobial agents especially when the prevalence of resistant strains is as high as what we reported here. In low-income countries like Ethiopia, where alternative therapeutic options are limited, it demands the use of available drugs with more in-depth knowledge of the strains in clinical setups and community. Because these strains are resistant to several classes of antimicrobials prescribed in the country, reconsidering the existing or development of strict prescription policy or revising treatment guidelines may be desirable. It requires a concerted effort of local/health institutions, national authorities and all other stake holders.

Furthermore, the detection of several high-risk international clones at the study site necessitates establishing centers for surveillance and tracking of epidemic clones and strengthening where available. Besisdes, strengthening infection prevention centers and adapting control strategies may be required together with strong principles and practices of antimicrobial stewardship.

Finally, strengthening existing capacity of microbiology laboratories, developing innovative strategies to combat antimicrobial resistance is critically important at this stage. Coordination of local resources, knowledge, and collaboration from local to global is highly desirable. More commitment and enagement of professionals from clinical, public health and policy makers is needed more than ever.

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ANNEXES

ANNEX-I: Information sheet for adult participants

Annex-I-A: Amharic version

ለጥናቱ ተሳታፊ አዋቂዎች የመረጃ ገፅ

ርዕስ ፡ የባክቴርያን ስርጭትና መድሃኒት የመላመድ ባህሪ የዘረ መል ጥናት

መግቢያ፡ ይህ ጥናት የሚካሄደው በጅማ ዩኒቨርሲቲ ሆስፒታል በሚታከሙ ህመምተኞች ላይ ሲሆን የሚካሄደው ጥናት ባክቴርያ ተብለው የሚጠሩ ተሕዋሲያንን መለየትና እንድሁም መድሃኒ ለመላመዳቸው ምክንያት የሆነውን የዘረ መል ዓይነትና ሥርጭት ለማወቅ ነው፡፡

የጥናቱ ዓላማ፡ የጥናቱ ዓላማ እነዚህን ተህዋሲያን በባህሪቸዉና በሥርጭታቸው ከተለዩ በኃላ በጥናቱ ላይ ለተሳተፉ ሕሙማንም ሆነ አጠቃላይ የሆስፒታሉን አገልግሎት እንዲሻሻል የመፍትሄ አቅጣጫ ለመጠቆም ይረዳል፡፡

የጥናቱ ተሳታፊዎች ሐላፊነት፡ ከጥናቱ ተሳታፊዎች የሚጠበቀው ለጥናቱ የሚፈለገዉን ናሙና እና መረጃ መስጠት/መፍቀድ ነው፡፡ይህም ናሙና ሽንት፣ሠገራ፣ህክታ፣ደም፣የህበለሠረሠር ፈሳሽ ሊሆን ይችላል፡፡ የደም ናሙና ከሆነ ተሳታፊዎቹ እንደ እድሜያቸው ማለትም ጨቅላ ሕፃናት 01 ሚሊ ሊትር፣ ከአምስት ዓመት በታች ከ3-5ሚሊሊትር፣ ከአምስት ዓመት በላይ ከ7-10 ሚሊ ሊተር ደም መስጠት አለባቸው፡፡ ከህብለሠረሠር የሚቀዳ ፈሳሽ ከሆነ በሁሉም እድሜ ክልል የሚገኙ ተሳታፊዎች 01 ሚሊ ሊተር መስጠት አለባቸው፡፡ በመጨረሻም ለዚህ ምርምር የሚወሰድ ናሙና ልማንኛዉም ህመምተኛ በየዕለቱ ከሚወሰዱ ናሙናዎች የተለየ አይደለም፡፡ ሆኖም የደም ናሙና እና የህብለሠረሠር ፈሣሽ ሲወሰድ ህመም ሊኖረው ይችላል፡፡

ከጥናቱ ጋር የተያያዘ ጉዳት፡ በጥናቱ ላይ በመሳተፍዎ ሊደርስ የሚችል የተለየ ጉዳት የለም፡፡ ነገር ግን እንደ ማንኛዉም የላቦራቶሪ ምርመራ ናሙና በመርፌ ሲወሰድ መጠነኛ ህመም ሊኖረው ይችላል፡፡

ከጥናቱ የሚገኝ ጥቅም፡ በዚህ ጥናት ላይ በመሳተፍዎ የሚያገኙት ቀጥተኛ ጥቅም የለም፡፡ ይሁን እንጂ በጥናቱ የሚገኝ የምርመራ ዉጤት ለእርስዎ ሕክምና ጠቃሚ ሆኖ ከተገኘ ጤናዎን ለሚከታተል ሐኪም ይገለፃል፡፡ ከዚህ ባለፈ ግን በአጠቃላይ ለህብረተሰብ ከፍተኛ ጥቅም አለው፡፡

ሚስጢር መጠበቅ፡ የእርስዎ የትኛዉም መረጃ በጥብቅ ሚስጢር ይያዛል፡፡ ለሌላ ሶስተኛ ወገን እርስዎን ማንነት በሚገልጥ መልኩ አይሰጥም፡፡

ሙብት ፡ የትናቱ ተሳታፊዎች የሚከተሉትን ሙብቶች አላቸው፡፡

መረጃ ያለመስጠት መብት

በጥናቱ ዉስጥ ለመሳተፍ መብት

ናሙና ያለመስጠት መብት

በጥናቱ ላይ ባለመሳተፍዎ የሚከለከሉት የትኛዉም የሕክምና አገልግሎት የለም፡፡

የጥናቱ ተጠሪዎች

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1. አዲስ አባባ ዩኒቨርሲቲ

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Annex-I-B: English version

Title: Epidemiology, molecular characteristics, and antimicrobial susceptibility pattern of ESBL producing Gram-negative bacilli (*Enterobacteriaceae* and *Pseudomonas*) among patients visiting Jimma Teaching Hospital**,** Ethiopia.

1. **Background**

   The study is designed to determine the Epidemiology and Molecular characteristics of Extended Spectrum β-lactamase producing gram negative bacilli among patients visiting JUTH. The study will reveal the current situation, novel information about ESBL producing gram negative bacilli and antimicrobial susceptibility to other antimicrobial agents too.

2. **Purpose of the study**

   The study is designed to determine the bacterial profile, ESBL phenotypes, genotypes and clonal types and susceptibility to other antimicrobial agents.

3. **Role of Participant**

   Study participants are expected to be volunteer for the specimen collection, give socio-demographic and clinical data. These specimens could be stool, urine, wound swab, blood, CSF and spurum. If the specimen is blood you should give 10ml blood. In case of CSF you should give 1ml of CSF.

4. **Risk associated with participation**

   The anticipated risk associated with specimen collection and laboratory investigation is minimal. During specimen collection by needles (blood and CSF) there might be minimal discomfort. This is similar to the specimen collection for the routine laboratory diagnosis.

   Benefits

   There is no direct benefit for study participant because of your participation to this study. However, if the result of the investigation is found beneficial (bacterial profile, AST) for clinical management of your case, it may be communicated to the physician attending the case. On the other hand, the final output of the research report will be used to improve the prescription, drug selection, and management of the infections caused by gram negative bacteria at JUTH. It may be used to develop new treatment

guidelines working this hospital. For policy makers it will help to consider the epidemiology of drug resistance in this area.

5. **Confidentiality**
   All the information obtained from you will be kept confidential. It will never be shared with other individuals including identifiers.
6. **Right**
   The participants have the right to
   - Keep hold of information
   - Decline to participate in the study
   - With draw from the study, this would have no bearings at all on their health benefits
   - Refuse provision of specimen
7. **Whom to contact**
   The study participants/parents/guardians/have full right to ask information about the research before they decide to participate; you can contact the principal investigator for any doubts that you want to clear.

1. Tsegaye Sewunet (PI)
   Tel. 091-171-8518
   E- mail: tsgishs2010@gmail.com
2. Dr. Daniel Asrat (Supervisor)
   Tel: 091-122-3019
   E-mail: asratdan@gmail.com
3. The Institutional Review Board (IRB)

Addis Ababa University
College of Health Sciences
Tel. 011-8961-396
E- mail: aaumirb@yahoo.com

4. AHRI (Armaour Hansen Research Institute)
   Tel: +251-11-321-1334
   E-mail: ahri@ethionet.et

Annex-I-C: Afaan oromoo version

Fuula odeesfannoo (namoota qorannoon kun irratti gaggeefamuuf)

Mata-duree: Baaakteeriyaa dhukubba fiduun beekamani irratti waa'ee amala dawaan wal-baruu fi faca'ina baakteeriyaa kanaa haangam akka ta'e baruuf qorannaa gageefamu.

1. **Seensa:** Qorannoon kun kan gaggeefamu dhukubfatoota hospitaala yuniversitii jimmaatti yaalaman irratti yeroo ta'u; baakteeriyaa dhukkuba fiduu danda'ani faca'ina isaanii fi dawaa waliin wal-baruu baakteeriyaa kanaa sadarkaa giinii ti qorachuu dha.

2. **Kaayyoo qorannoo kanaa:** baakteeriyaan dhukuba fiduu danda'ani kana amala isaanii, faca'ina isaanii, fi gosa gosa isaanii maal akka ta'an erga baramee booda; dhukubfatoota qorannoo kana keessatti hirmaannaa qabaniifis ta'e waliigala tajaajila hospital katti kennamu fooyyessuuf kallattii furmaata ni mul'isa.

3. **Dirqama namoota qorannoo kana keessa jiranii:** Jarri qorannoo kanaa hirmaatan qorannoo kanaaf waan barbaachisu iddattoo kennuu qabu. Idaattoon kun boolii guddaa, boolii xiqqaa, hakkee, dhiiga, akkasumas dhangalaa'aa lafee dugdaa keesssaa ta'uu danda'a. Dhiiga yoo ta'e mililitra 07-10 kennuu qabu. Yoo dhangala'aa lafee dugdaa keessaa fuudhamu ta'e miliilitra 01kennuu qabu.

4. **Miidhaa:** qorannoo kana irraatti waan hirmaataniif miidhaan isin irra gahu hinjiru. Haata'u malee, akuma qorannoo laboratoorii idilee ti yoo diigi ni fuudhama ta'e yeroo lilmoon isin waraantu dhukkubbii xiqqoon yeroo sanaaf isnitti dhagahamuu danda'a.

5. **Faaydaa:** qorannoo kana irraattii sababa hiraataniif kallatiidhaan faaydaan isn argattan hinjiru. Haata'u malee, friin qorannoo kanaa tajaajila wal'aansaa isiniif kennamu irraatti bu'aa qabeessa yoo ta'e ogeessa fayyaa keessanii hordofaa jrutti ni himama. Karaa biraa walumaa gala faydaa qorannoo kanaa akka hawaasaatti tajaajila fayyaa hawaasaa foyyeessuu irrattii bu'aa qaba.

6. **Iccitii eeguu:** walumaa gala odeefannoon isn nuuf kennitan kun bu'aan qorannoo kana hundi iccitiin ni qabama. Namni kan biraa kam iyyuu beekuu hin danda'u.

7. **Mirga:** yeroo qorannoo kana irratti hirmaatan mirga guutuu armaan gaditti tarreefame qabdu

    - Odeefannoo kennuu dhiisuuf
    - Qorannoo kana irraatti hirmaachuu diisuu
    - Iddattoo kennuu dhiisuu

- Kunis mirga tajaajila hospitaala kana itti fayadamu qabdan kamillee irraa isin hindangessu.

8. Itti gaaftamaa qorannoo kanaa:

8.1.Tseggayee Sewunnet (Qorataa- dursaa)
Lakk. Bil: 091-171-8518
E-mail: tsegishs2010@gmail.com

8.2.Dr. Daani'eel Asraat (To'ataa tokkoffaa)
Lakk. Bil: 091-122-3019
E-mail: asratdan@gmail.com

8.3.Gumii dhaabbata xiinxala fi naamusa qorannoo

8.3.1. Addis Ababa Yuniversiitii
Kooleejji Barnoota Fayyaa
Lakk.Bil: +251-118-961-396
E-mail: aaumirb@yahoo.com

8.3.2. Dhaabata qorannoo 'AHRI'
Karaa Jimmaa irra
L.S.P. 1005
Lakk.bil. +251-11-321-1334
E-mail: ahri@ethionet.et
A.A

ANNEX-II: Consent form for adult participants

Annex-II-A: Amharic Version

የአማርኛ የስምምነት ቅፅ

የምስጢር ቁጥር ----------

ተሳታፊው ሙሉ ስም ----------

እኔ ስሜ ከላይ የተጠቀሰው የባክቴርያን ስርጭትና መድሃኒት የመላመድ ባህሪ የዘረ መል ጥናት ሊደረግ በታቀደው ጥናት ላይ እንድሳተፍ ተጠይቄ ስለ ጉዳዩ ለመረዳት በቂ መረጃ አግኝቻለሁ፡፡ ስለሆነም ለጥናቱ የሚሆን ናሙና እንዲወሰድ የደም ናሙና ከሆነ ከ7-10 ሚሊ ሊተር ደም መስጠት አለባቸው እንዲሁም ከህብለሠረሠር የሚቀዳ ፈሳሽ ከሆነ 01 ሚሊ ሊተር ለመስጠት ፈቃደኛ ነኝ፡፡ ስለዚህ በጥናቱ ለመሳተፍ ፈቃደኛ መሆኔን እገልፃለሁ፡፡ በምርምሩ መሳተፍ ወይም አለመሳተፍ መብቴ የተጠበቀ መሆኑን እና ላለመሳተፍ ብውሰን በሆስፒታል በሚደረግልኝ ሕክምና ላይ ምንም ተፅእኖ እንደሌለው ተርድቻለሁ፡፡

ስለዚህ የጥናቱን ጠቃሚነት ተረድቼ አምኜበት የስምምነት ቃሌን የሰጠሁት በፍፁም ፈቃደኝነት ነው፡፡ አንዳንድ ማብራሪያ የሚፈልጉ ጥያቄዎችን ጠይቄ መልስ ተሰጥቶኛል፡፡

በዚህም መሰረት በፈቃዴ መስማማቴን በፊርማዬ አረጋግጣለሁ፡፡

የተሳታፊው ፊርማ	የምስክር ፊርማ
----------	----------

Annex-II-B: English version

Clients ID_____
Name of the client _____

I have been informed about the study titled "Epidemiology and Molecular characteristics of ESBL producing gram negative bacteria among patients visiting JUTH". The objective and application of the study were explained to me. I am also informed that all information contained within the questionnaire is to be kept confidential. Moreover, I have also been well informed of my right to keep hold of information, decline to cooperate and drop out of the study if I want and none of my actions will have any bearing at all on my overall health care and hospital access.

It is therefore with full understanding of the situations that I agreed to give the written consent voluntarily to the researcher to give specimen (if the specimen is blood, 7-10ml and if CSF 01ml). Inaddition I have had the opportunity to ask a question about the project and I have got the clarification to my satisfaction.

Furthermore, I was also told that results will be reported timely to physician in charge for appropriate treatment and management of my case. And hence I agreed that I am contributing to the treatment of my fellows and myself by contributing in this project.

I _____ the undersigned hereby give my consent for giving the requested information and specimen for the purpose of the study mentioned above.

Participant's Signature: ----------
Witness' Signature ----------

Date ------/-------/----

Annex-II-C: Afaan oromoo Version

Waliigaltee (qorannoo irratti hirmaachuuf)

Koodii _____
Maqaa Qoratamaa_____

Ani maqaan koo armaan olitti kan ibsame qorannoo baakteeriyaa dhukuba fidan faca'ina isaaniitiif dawaan walbaruu isaanii irratti akka hirmaadhu gaafatamee, waa'ee qoranichaas odeefannoo gahaa argadheera. Kanaaf qorannoo kan irratti hirmaachuun iddattoo qorannoo kanaaf barbaachisu (dhiiga yoo ta'e mililitra 07-10 kennuu qabu. Yoo dhangala'aa lafee dugdaa keessaa fuudhamu ta'e miliilitra 01) akkan kennu fedha koo ta'uu ibseera. Ani qorannoo kana irratti hirmaachuuf fedha guutuun walii galuu koo nan ibsa.

Qorannoo kana irratti hirmaachuuf /dhiisuun mirga koo akka ta'e baree, yoon hirmaachuu dhiise tajaajila hospitaala kana keessaa argadhus ta'e, kan biro kamiyyuu irratti dhiibbaa akka hin qabne hubadheera. Gaafiiwaan tokko tokko gaafadhee deebii quubsaan naaf kennameera.

Kanaaf shakkii tokko malee hubannaa sirrii argadhee, irraatti walii galuu koo mallattoo koon nan mirkaneessa.

Hirmaataa :
Maqaa_____
Mallattoo _____

Mallattoo Ragaa

Maqaa _____
Mallattoo_____

Ragaa

Maqaa _____
Mallattoo _____

ANNEX-III: Information sheet for parents/guardians

Annex-III-A: Amharic Version

በጥናቱ ተሳታፊ ለሆኑ ሕፃናት ወላጆ ወይም አሳዳጊዎች የመረጃ ገፅ

ርዕስ ፡ የባክቴርያን ስርጭትና መድሃኒት የመላመድ ባህሪ የዘረ መል ጥናት

መግቢያ፡ ይህ ጥናት የሚካሄደው በጅማ ዩኒቨርሲቲ ሆስፒታል በሚታከሙ በሽተኞች ላይ ሲሆን የሚካሄደው ጥናት ባክቴርያ ተብለው የሚጠሩ ተሕዋሲያንን መለየትና እንድሁም መድሃኒት ለመላመዳቸው ምክንያት የሆነውን የዘረ መል ዓይነትና ሥርጭት ለማወቅ ነው፡፡

የጥናቱ ዓላማ፡ የጥናቱ ዓላማ እነዚህን ተህዋሲያን በባህሪቸዉና በሥርጭታቸው ከተለዩ በኃላ በጥናቱ ላይ ለተሳተፉ ሕሙማንም ሆነ አጠቃላይ የሆስፒታሉን አገልግሎት እንዲሻሻል የመፍትሄ አቅጣጫ ለመጠቆም ይረዳል፡፡

የጥናቱ ተሳታፊዎች ሐላፊነት፡ ከጥናቱ ተሳታፊዎች የሚጠበቀው ለጥናቱ የሚፈለገዉን ናሙና እና መረጃ መስጠት/መፍቀድ ነው፡፡ ይህም ናሙና ሽንት፣ሠገራ፣ህክታ፣ደም፣የህበለሠረሠር ፈሳሽ ሊሆን ይችላል፡፡ የደም ናሙና ከሆነ ተሳታፊዎቹ እንደ እድሜያቸው ማለትም ጨቅላ ሕፃናት 01 ሚሊ ሊትር፣ ከአምስት ዓመት በታች ከ3-5ሚሊሊትር፣ ከአምስት ዓመት በላይ ከ7-10 ሚሊ ሊተር ደም መስጠት አለባቸው፡፡ ከህብለሠረሠር የሚቀዳ ፈሳሽ ከሆነ በሁሉም እድሜ ክልል የሚገኙ ተሳታፊዎች 01 ሚሊ ሊተር መስጠት አለባቸው፡፡

ከጥናቱ ጋር የተያያዘ ጉዳት፡ የወለዱት ወይም ያሳደጉት ልጅዎ በጥናቱ ላይ ቢሳተፍ/ብትሳተፍ ሊደርስ የሚችል ዝቀተኛ ነዉ፡፡ ነገር ግን እንደ ማንኛዉም የላቦራቶሪ ምርመራ ናሙና በመርፌ (ደም ወይም የህበለሰረሰር ፈሳሽ) ሲወሰድ መጠነኛ ህመም ሊኖረው ይችላል፡፡

ከጥናቱ የሚገኝ ጥቅም፡ የወለዱት ወይም ያሳደጉት ልጅዎ በጥናቱ ላይ ቢሳተፍ/ብትሳተፍ የሚያገኙት ቀጥተኛ ጥቅም የለም፡፡ ይሁን እንጂ በጥናቱ የሚገኝ የምርመራ ዉጤት ለልጅዎ ሕክምና ጠቃሚ ሆኖ ከተገኘ ጤናዉን ለሚከታተል ሐኪም ይገለፃል፡፡ከዚህ ባለፈ ግን በአጠቃላይ ለህብረተሰብ ከፍተኛ ጥቅም አለው፡፡

ሚስጢር መጠበቅ፡ የእርስዎም ሆነ የልጅዎ የትኛዉም መረጃ በጥብቅ ሚስጢር ይያዛል፡፡ ለሌላ ሶስተኛ ወገን እርስዎን ወይም የልጅዎን ማንነት በሚገልጥ መልኩ አይሰጥም፡፡

መብት ፡ የትናቱ ተሳታፊዎች የሚከተሉትን መብቶች አላቸው፡፡

መረጃ ያለመስጠት መብት

በጥናቱ ዉስጥ ለመሳተፍ መብት

ናሙና ያለመስጠት መብት

በጥናቱ ላይ ባለመሳተፍዎ የሚከለከሉት የትኛዉም የሕክምና አገልግሎት የለም፡፡

የጥናቱ ተጠሪዎች

ፀጋዬ ሰዉነት (ተመራማሪ)

ስልክ፤ 091-171-8518

ኢ. ሜይል፡ tsegishs2010@gmail.co.

ዶ/ር ዳንኤል አስራት (ሱፐርቫይዘር)

ስልክ፡ 091-122-3019

ኢ.ሜይል፤ asratdan@gmail.com

ተቋማዊ የምርምር ስነምግባርና ክትትል ጉባኤ

አዲስ አባባ ዩኒቨርሲቲ

ሕክምና ሳይንስ ኮሌጅ

ስልክ፤ 011-896-1396

ኢ.ሜይል፡ aaumirb@yahoo.com

አህሪ/አለርት የምርምር ተቋም

Jimma road:

Po.box 1005

Tel.no: +251-11-321-1334

Annex-III-B: English version

Title: Epidemiology, molecular characteristics, and antimicrobial susceptibility patern of ESBL producing Gram-negative bacilli (*Enterobacteriaceae* and *Pseudomonas*) among patients visiting Jimma Teaching Hospital**,** Ethiopia.

1. Background

The study is designed to determine the Epidemiology and molecular characteristics of ESBL producing gram negative bacilli among patients visiting JTH. The study will reveal the current situation, novel information about ESBL producing gram negative bacilli and antimicrobial susceptibility to other antimicrobial agents too.

2. Purpose of the study

The study is designed to determine the bacterial profile, ESBL phenotypes, genotypes and clonal types and susceptibility to other antimicrobial agents.

3. Role of Participant

Study participants are expected to be volunteer for the specimen collection, give socio-demographic and clinical data. These specimens could be stool, urine, wound swab, blood, CSF and spurum. If the specimen is blood you should give 10ml blood. In case of CSF you should give 1ml of CSF.

4. Risk associated with participation

The anticipated risk associated with specimen collection and laboratory investigation is minimal. During specimen collection by needles there might be minimal discomfort. This is like the specimen collection for the routine laboratory diagnosis.

Benefits

There is no direct benefit for study participant because of your participation to this study. However, if the result of the investigation is found beneficial (bacterial profile, AST) for clinical management of your case, it may be communicated to the physician attending the case. On the other hand, the final output of the research report will be used to improve the prescription, drug selection, and management of the infections caused by gram negative bacteria at JUTH. It may be used to develop new treatment guidelines working for this hospital. For policy makers it will help to consider the epidemiology of drug resistance in this area.

5. Confidentiality

All the information obtained from you will be kept confidential. It will never be shared with other individuals with identifiers.

6. Right

The participants have the right to

- Keep hold of information
- Decline to participate in the study
- With draw from the study, this would have no bearings at all on their health benefits
- Refuse provision of specimen

7. Whom to contact

The study participants/parents/guardians/have full right to ask information about the research before they decide to participate; you can contact the principal investigator for any doubts that you want to clear.

1. Tsegaye Sewunet (PI)
   Tel. 091-171-8518
   E- mail: tsgishs2010@gmail.com
2. Dr. Daniel Asrat (Supervisor)
   Tel: 091-122-3019
   E-mail: asratdan@gmail.com
3. The Institutional Review Board (IRB)

3.1. Addis Ababa University
College of Health Sciences
Tel. 011-896-1396
E- mail: aaumirb@yahoo.com

3.2. AHRI (Armaour Hansen Research Institute)
Tel: +251-11-321-1334
E-mail:

Annex-II-C: Afaan oromoo version

Fuula odeesfannoo (maatii daa'iman qorannoo kana irratti hirmaataniif)

Mata-duree: Baaakteeriyaa dhukubba fiduun beekamani irratti waa'ee amala dawaan wal-baruu fi faca'ina baakteeriyaa kanaa haangam akka ta'e baruuf qorannaa gageefamu.

Seensa: Qorannoon kun kan gaggeefamu dhukubfatoota hospitaala yuniversitii jimmaatti yaalaman irratti yeroo ta'u; baakteeriyaa dhukkuba fiduu danda'ani faca'ina isaanii fi dawaa waliin wal-baruu baakteeriyaa kanaa sadarkaa giinii ti qorachuu dha.

Kaayyoo qorannoo kanaa: baakteeriyaan dhukuba fiduu danda'ani kana amala isaanii, faca'ina isaanii, fi gosa gosa isaanii maal akka ta'an erga baramee booda; dhukubfatoota qorannoo kana keessatti hirmaannaa qabaniifis ta'e waliigala tajaajila hospital katti kennamu fooyyessuuf kallattii furmaata ni mul'isa.

Dirqama namoota qorannoo kana keessa jiranii: daa'iman qorannoo kanaa hirmaatan qorannoo kanaafwaan barbaachisu iddattoo kennuu qabu. Idaattoon kun boolii guddaa, boolii xiqqaa, hakkee, dhiiga, akkasumas dhangalaa'aa lafee dugdaa keesssaa ta'uu danda'a. Dhiiga yoo ta'e daa'imi ji'a tokkoo gadii miliilitra tokko, ijoolleenb ji'a tokkoo ol hanga waggaa shanii mililitra 3-5, waggaa shanii ol kan jiran mililitra 7-10; yoo dhangala'aa lafee dugdaa keessaa ta'e immoo miliilitra 01 kennuu qabu.

Miidhaa: qorannoo kana irraatti waan hirmaataniif miidhaan daa'ima keessan irra gahu hinjiru. Haata'u malee, akuma qorannoo laboratoorii idilee yoo diigini fuudhama ta'e yeroo lilmoon waraantu dhukkubbii xiqqoon yeroo sanaaf itti dhagahamuu danda'a.

Faaydaa: qorannoo kana irraattii sababa hiraataniif kallatiidhaan faaydaan argattan hinjiru. Haata'u malee, friin qorannoo kanaa tajaajila wal'aansaa isiniif kennamu irraatti bu'aa qabeessa yoo ta'e ogeessa fayyaa keessanii hordofaa jrutti ni himama. Karaa biraa walumaa gala faydaa qorannoo kanaa akka hawaasaatti tajaajila fayyaa hawaasaa foyyeessuu irrattii bu'aa qaba.

Iccitii eeguu: walumaa gala odeefannoon nuuf kennitan kun bu'aan qorannoo kana hundi iccitiin ni qabama. Namni kan biraa kam iyyuu beekuu hin danda'u.

Mirga: yeroo qorannoo kana irratti hirmaatan mirga guutuu armaan gaditti tarreefame qabdu

- Odeefannoo kennuu dhiisuuf
- Qorannoo kana irraatti hirmaachuu diisuu
- Iddattoo kennuu dhiisuu

- Kunis mirga tajaajila hospitaala kana itti fayadamu qabdan kamillee irraa isin hindangessu.

Itti gaaftamtoota qorannoo kanaa:

Tseggayee Sewunnet (Qorataa- dursaa)
Lakk. Bil: 091-171-8518
E-mail: tsegishs2010@gmail.com
Dr. Daani'eel Asraat (To'ataa tokkoffaa)
Lakk. Bil: 091-122-3019
E-mail: asratdan@gmail.com
Gumii dhaabbata xiinxala fi naamusa qorannoo
Addis Ababa Yuniversiitii
Kooleejji Barnoota Fayyaa
Lakk.Bil: 011-896-1396
E-mail: aaumirb@yahoo.com
Dhaabata qorannoo 'AHRI'
- Karaa Jimmaa irra
  L.S.P. 1005
  Lakk.bilbil. +251-11-321-1334

ANNEX-IV: Consent for parents/guardians

IV-A: Amharic Version

የአማርኛ የስምምነት ቅፅ

የምስጢር ቁጥር ----------

የተሳታፊው ሙሉ ስም ----------

የቤተሰብ ወይም አሳዳጊ ሙሉ ስም ----------

ዝምድና ----------

እኔ ስሜ ከላይ የተጠቀሰው የባክቴርያን ስርጭትና መድሃኒት የመላመድ ባህሪ የዘረ መል ጥናት ሊደረግ በታቀደው ጥናት ላይ ልጄ እንዲሳተፍ/እንድትሳተፍ ተጠይቄ ስለ ጉዳዩ ለመረዳት በቂ መረጃ አግኝቻለሁ፡፡ ስለሆነም ለጥናቱ የሚሆን ናሙና የደም ናሙና ከሆነ ተሳታፊዎቹ እንደ እድሜያቸው ማለትም ጨቅላ ሕፃናት 01 ሚሊ ሊትር፣ ከአምስት ዓመት በታች ከ3-5ሚሊሊትር፣ ከአምስት ዓመት በላይ ከ7-10 ሚሊ ሊተር እንዲሁም ከህብለሠረሠር የሚቀዳ ፈሳሽ ከሆነ 01 ሚሊ ሊተር ቢወሰድ እናም ልጄ ወይም የማሳድገው/ጋት ልጄ በጥናቱ እንዲሳተፍ/እንድትሳተፍ ሙሉ ፈቃደኛ መሆኔን እገልፃለሁ፡፡ ከምርምሩ መሳተፍ ወይም አለመሳተፍ ሙቤቴ የተጠበቀ መሆኑን እና ላለመሳተፍ ብውስን በሆስፒታል በሚደረግልን ሕክምና ላይ ምንም ተፅእኖ እንደሌለው ተርድቻለሁ፡፡

ስለዚህ የጥናቱን ጠቃሚነት ተረድቼ አምኜበት የስምምነት ቃሌን የሰጠሁት በፍፁም ፈቃደኝነት ነው፡፡ በመጨረሻም አንዳንድ ማብራሪያ የሚፈልጉ ጥያቄዎችን ጠይቄ መልስ ተሰጥቶኛል፡፡

በዚህም መሰረት በፈቃዴ መስማማቴን በፊርማዬ አረጋግጣለሁ፡፡

የቤተሰብ ወይም አሳዳጊ ፊርማ የምስክር ፊርማ

---------- ----------

Annex-IV-B: English version

Clients ID_____
Name of the client _____
Name of the family/guardian _____
Relationship to the participant _____

I have been informed about the study titled "Epidemiology and molecular characteristics of ESBL producing Gram-negative bacilli among patients visiting JUTH". The objective and application of the study were explained to me. I am also informed that all information contained within the questionnaire is to be kept confidential. Moreover, I have also been well informed of my right to keep hold of information, decline to cooperate and drop out of the study if I want and none of my actions will have any bearing at all on my overall health care and hospital access.

I agreed to give this written consent voluntarily to the researcher to give specimen from my child (If the specimen is blood (from neonates 1ml, from from children under age of five 5ml, above five years 7-10ml of blood and if the specimen is CSF 01ml for all age group). Inaddition, I have had the opportunity to ask a question about the study and I am clarified to my satisfaction.

Furthermore, I was also told that results will be reported timely to physician in charge for better management of the condition my child.

I _____ the undersigned hereby give my consent for giving the requested information and specimen for the purpose of the study mentioned above.

Signature (participant/parent/ guardian): ----------
Witness (Illiterate) ----------
Physician/principal investigator/data collector ----------

Date ------/-------/----

Annex-IV-C: Afaan oromoo Version

Waliigaltee (qorannoo irratti hirmaachuuf)

Koodii _____
Maqaa Qoratamaa_____
Maqaa Abbaa/Guddisaa_____
Firooma qaban_____

Ani maqaan koo armaan olitti kan ibsame, qorannoo baakteeriyaa dhukuba fidan, faca'ina isaaniitiif dawaan walbaruu isaanii qorachuuf gaggeefanmu irratti daa'mi koo/daa'imi ani gudisu akka hirmaatu/ttu gaafatamee, waa'ee qoranichaas odeefannoo gahaa argadheera. Kanaaf qorannoo kan irratti hirmaachuun iddattoo qorannoo kanaaf barbaachisu (dhiiga yoo ta'e daa'imi ji'a tokkoo gadii miliilitra tokko, ijoolleenb ji'a tokkoo ol hanga waggaa shanii mililitra 3-5 waggaa shanii ol kan jiran mililitra 7-10; yoo dhangalaa'aa lafee dugdaa keessaa ta'e miliilitra 01) akkan kennu gaafatamee fedha koo ta'uu ibseera. Mucaan koo (kanan dahe/dhashe)/yookis guddisu qorannoo kana irratti hirmaachuuf fedha guutuun walii galuu koo nan ibsa.

Qorannoo kana irratti hirmaachuu ykn dhiisuun mirga akka ta'e baree, yoon hirmaachuu dhiise tajaajila hospitaala kana keessaa argadhus ta'e, kan biro kamiyyuu irratti dhiibbaa akka hin qabne hubadheera.

Haaluma kanaan faaydaa qorannoo kanaa hubadhee, itti amanee, fedha guutuun irratti hirmaachuuf murteeseera. Gaafiiwaan tokko tokko gaafadhee deebii quubsaan naaf kennameera.

Kanaaf shakkii tokko malee hubannaa sirrii argadhee, irraatti walii galuu koo mallattoo koon nan mirkaneessa.

Mallattoo Abbaa/Guddisaa _____
Maqaa_____

Ragaa

Maqaa _____
Mallattoo _____

ANNEX-V: Information sheet for 12-17 years old

Annex-V-A: Amharic version

ለጥናቱ ተሳታፊ ሕፃናት የመረጃ ገፅ

ርዕስ ፡ የባክቴርያን ስርጭትና መድሃኒት የመላመድ ባህሪ የዘረ መል ጥናት

መግቢያ፡ ይህ ጥናት የሚካሄደው በጅማ ዩኒቨርሲቲ ሆስፒታል በሚታከሙ ህመምተኞች ላይ ሲሆን የሚካሄደው ጥናት ባክቴርያ ተብለው የሚጠሩ ተሕዋሲያንን መለየትና እንድሁም መድሃኒት ለመላመዳቸው ምክንያት የሆነውን የዘረ መል ዓይነትና ሥርጭት ለማወቅ ነው፡፡

የጥናቱ ዓላማ፡ የጥናቱ ዓላማ እነዚህን ተህዋሲያን በባህሪቸዉና በሥርጭታቸው ከተለዩ በኃላ በጥናቱ ላይ ለተሳተፉ ሕሙማንም ሆነ አጠቃላይ የሆስፒታሉን አገልግሎት እንዲሻሻል የመፍትሄ አቅጣጫ ለመጠቆም ይረዳል፡፡

የጥናቱ ተሳታፊዎች ሐላፊነት፡ ከጥናቱ ተሳታፊዎች የሚጠበቀው ለጥናቱ የሚፈለገዉን ናሙና እና መረጃ መስጠት/መፍቀድ ነው፡፡ይህም ናሙና ሽንት፣ሠገራ፣ህክታ፣ደም፣የህበለሠረሠር ፈሳሽ ሊሆን ይችላል፡፡ የደም ናሙና ከሆነ ተሳታፊዎቹ እንደ እድሜያቸው ማለትም ጨቅላ ሕፃናት 01 ሚሊ ሊትር፣ ከአምስት ዓመት በታች ከ3-5ሚሊሊትር፣ ከአምስት ዓመት በላይ ከ7-10 ሚሊ ሊተር ደም መስጠት አለባቸው፡፡ ከህበለሠረሠር የሚቀዳ ፈሳሽ ከሆነ በሁሉም እድሜ ክልል የሚገኙ ተሳታፊዎች 01 ሚሊ ሊተር መስጠት አለባቸው፡፡ በመጨረሻም ለዚህ ምርምር የሚወሰድ ናሙና ለማንኛዉም ህመምተኛ በየዕለቱ ከሚወሰዱ ናሙናዎች የተለየ አይደለም፡፡ ሆኖም የደም ናሙና እና የህበለሠረሠር ፈሣሽ ሲወሰድ ህመም ሊኖረው ይችላል፡፡

ከጥናቱ ጋር የተያያዘ ጉዳት፡ በጥናቱ ላይ በመሳተፍዎ ሊደርስ የሚችል የተለየ ጉዳት የለም፡፡ ነገር ግን እንደ ማንኛዉም የላቦራቶሪ ምርመራ ናሙና በመርፌ ሲወሰድ መጠነኛ ህመም ሊኖረው ይችላል፡፡

ከጥናቱ የሚገኝ ጥቅም፡ በዚህ ጥናት ላይ በመሳተፍዎ የሚያገኙት ቀጥተኛ ጥቅም የለም፡፡ ይሁን እንጂ በጥናቱ የሚገኝ የምርመራ ዉጤት ለእርስዎ ሕክምና ጠቃሚ ሆኖ ከተገኘ ጤናዎን ለሚከታተል ሐኪም ይገለፃል፡፡ ከዚህ ባለፈ ግን በአጠቃላይ ለህብረተሰብ ከፍተኛ ጥቅም አለው፡፡

ሚስጢር መጠበቅ፡ የእርስዎ የትኛዉም መረጃ በጥብቅ ሚስጢር ይያዛል፡፡ ለሌላ ሶስተኛ ወገን እርስዎን ማንነት በሚገልጥ መልኩ አይሰጥም፡፡

ሙቢት ፡ የጥናቱ ተሳታፊዎች የሚከተሉትን ሙብቶች አላቸው፡፡

መረጃ ያለመስጠት ሙብት

በጥናቱ ዉስጥ ለመሳተፍ መብት

ናሙና ያለመስጠት ሙብት

በጥናቱ ላይ ባለመሳተፍህ/ሽ የሚከለከል የትኛዉም የሕክምና አገልግሎት የለም፡፡

የጥናቱ ተጠሪዎች

ፀጋዬ ሰዉነት (ተመራማሪ)

ስልክ፤ 091-171-8518

ኢ. ሜይል፡ tsegishs2010@gmail.co.

ዶ/ር ዳንኤል አስራት (ሱፐርቫይዘር)

ስልክ፡ 091-122-3019

ኢ.ሜይል፤ asratdan@gmail.com

ተቋማዊ የምርምር ስነምግባርና ክትትል ጉባኤ

1. አዲስ አባባ ዩኒቨርሲቲ

ሕክምና ሳይንስ ኮሌጅ

ስልክ፤ 011-896-1396

ኢ.ሜይል፡ aaumirb@yahoo.com

2. አህሪ/አለርት የምርምር ተቋም

Po.box = 1005

Tel.no. +251-11-321-1334

ኢ.ሜይል፡ ahri@ethionet.et

Annex-V-B: English version

Title: Epidemiology, molecular characteristics, and antimicrobial susceptibility pattern of ESBL producing Gram-negative bacilli (*Enterobacteriaceae* and *Pseudomonas*) among patients visiting Jimma Teaching Hospital**,** Ethiopia.

8. **Background**

   The study is designed to determine the Epidemiology and Molecular characteristics of Extended Spectrum β-actamase producing gram negative bacilli among patients visiting JUTH. The study will reveal the current situation, novel information about ESBL producing gram negative bacilli and antimicrobial susceptibility to other antimicrobial agents too.

9. **Purpose of the study**

   The study is designed to determine the bacterial profile, ESBL phenotypes, genotypes and clonal types and susceptibility to other antimicrobial agents.

10. **Role of Participant**

    Study participants are expected to be volunteer for the specimen collection, give socio-demographic and clinical data. This specimen could be stool, urine, wound swab, blood, CSF and spurum. If the specimen is blood you should give 10ml blood. In case of CSF you should give 1ml of CSF.

11. **Risk associated with participation**

    The anticipated risk associated with specimen collection and laboratory investigation is minimal. During specimen collection by needles (blood and CSF) there might be minimal discomfort. This is similar to the specimen collection for the routine laboratory diagnosis.

    Benefits

    There is no direct benefit for study participant because of your participation to this study. However, if the result of the investigation is found beneficial (bacterial profile, AST) for clinical management of your case, it may be communicated to the physician attending the case. On the other hand, the final output of the research report will be used to improve the prescription, drug selection, and management of the infections caused by gram negative bacteria at JUTH. It may be used to develop new treatment

guidelines working this hospital. For policy makers it will help to consider the epidemiology of drug resistance in this area.

12. Confidentiality

All the information obtained from you will be kept confidential. It will never be shared with other individuals including identifiers.

13. Right

The participants have the right to

- Keep hold of information
- Decline to participate in the study
- With draw from the study, this would have no bearings at all on their health benefits
- Refuse provision of specimen

14. Whom to contact

The study participants/parents/guardians/have full right to ask information about the research before they decide to participate; you can contact the principal investigator for any doubts that you want to clear.

5. Tsegaye Sewunet (PI)
   Tel. 091-171-8518
   E- mail: tsgishs2010@gmail.com
6. Dr. Daniel Asrat (Supervisor)
   Tel: 091-122-3019
   E-mail: asratdan@gmail.com
7. The Institutional Review Board (IRB)

Addis Ababa University
College of Health Sciences
Tel. 011-8961-396
E- mail: aaumirb@yahoo.com

8. AHRI (Armaour Hansen Research Institute)
   Tel: +251-11-321-1334
   E-mail: ahri@ethionet.et

Annex-V-C: Afaan oromoo version

Fuula odeesfannoo (namoota qorannoon kun irratti gaggeefamuuf)

Mata-duree: Baaakteeriyaa dhukkuba fiduun beekamani irratti waa'ee amala dawaan wal-baruu fi faca'ina baakteeriyaa kanaa haangam akka ta'e baruuf qorannaa gageefamu.

1. **Seensa:** Qorannoon kun kan gaggeefamu dhukubfatoota hospitaala yuniversitii jimmaatti yaalaman irratti yeroo ta'u; baakteeriyaa dhukkuba fiduu danda'ani faca'ina isaanii fi dawaa waliin wal-baruu baakteeriyaa kanaa sadarkaa giinii ti qorachuu dha.
2. **Kaayyoo qorannoo kanaa:** baakteeriyaan dhukuba fiduu danda'ani kana amala isaanii, faca'ina isaanii, fi gosa gosa isaanii maal akka ta'an erga baramee booda; dhukubfatoota qorannoo kana keessatti hirmaannaa qabaniifis ta'e waliigala tajaajila hospital katti kennamu fooyyessuuf kallattii furmaata ni mul'isa.
3. **Dirqama namoota qorannoo kana keessa jiranii:** Jarri qorannoo kanaa hirmaatan qorannoo kanaaf waan barbaachisu iddattoo kennuu qabu. Idaattoon kun boolii guddaa, boolii xiqqaa, hakkee, dhiiga, akkasumas dhangalaa'aa lafee dugdaa keesssaa ta'uu danda'a. Dhiiga yoo ta'e mililitra 07-10 kennuu qabu. Yoo dhangala'aa lafee dugdaa keessaa fuudhamu ta'e miliilitra 01kennuu qabu.
4. **Miidhaa:** qorannoo kana irraatti waan hirmaataniif miidhaan isin irra gahu hinjiru. Haata'u malee, akuma qorannoo laboratoorii idilee ti yoo diigi ni fuudhama ta'e yeroo lilmoon isin waraantu dhukkubbii xiqqoon yeroo sanaaf isnitti dhagahamuu danda'a.
5. **Faaydaa:** qorannoo kana irraattii sababa hiraataniif kallatiidhaan faaydaan isn argattan hinjiru. Haata'u malee, friin qorannoo kanaa tajaajila wal'aansaa isiniif kennamu irraatti bu'aa qabeessa yoo ta'e ogeessa fayyaa keessanii hordofaa jrutti ni himama. Karaa biraa walumaa gala faydaa qorannoo kanaa akka hawaasaatti tajaajila fayyaa hawaasaa foyyeessuu irrattii bu'aa qaba.
6. **Iccitii eeguu:** walumaa gala odeefannoon isn nuuf kennitan kun bu'aan qorannoo kana hundi iccitiin ni qabama. Namni kan biraa kam iyyuu beekuu hin danda'u.
7. **Mirga:** yeroo qorannoo kana irratti hirmaatan mirga guutuu armaan gaditti tarreefame qabdu
    - Odeefannoo kennuu dhiisuuf
    - Qorannoo kana irraatti hirmaachuu diisuu
    - Iddattoo kennuu dhiisuu
    - Kunis mirga tajaajila hospitaala kana itti fayadamu qabdu kamillee irraa si hindangessu.
9. **Itti gaaftamaa qorannoo kanaa:**

    9.1.Tseggayee Sewunnet (Qorataa- dursaa)
    Lakk. Bil: 091-171-8518
    E-mail: tsegishs2010@gmail.com

    9.2.Dr. Daani'eel Asraat (To'ataa tokkoffaa)
    Lakk. Bil: 091-122-3019
    E-mail: asratdan@gmail.com

9.3.Gumii dhaabbata xiinxala fi naamusa qorannoo

9.3.1. Addis Ababa Yuniversiitii
Kooleejji Barnoota Fayyaa
Lakk.Bil: +251-118-961-396
E-mail: aaumirb@yahoo.com

9.3.2. Dhaabata qorannoo ‘AHRI’
Karaa Jimmaa irra
L.S.P. 1005
Lakk.bil. +251-11-321-1334
E-mail: ahri@ethionet.et
A.A

ANNEX-VI: Assent form

Annex-VI-A: Amharic Version

የአማርኛ የስምምነት ቅፅ

የምስጢር ቁጥር ----------

የህፃኑ ሙሉ ስም ----------

የቤተሰብ ወይም አሳዳጊ ሙሉ ስም ----------

ዝምድና ----------

እኔ ስሜ ከላይ የተጠቀሰው ህፃን የባክቴርያን ስርጭትና መድሃኒት የመላመድ ባህሪ የዘረ መል ጥናት ሊደረግ በታቀደው ጥናት ላይ እንድሳተፍ ተጠይቄ ስለ ጉዳዩ አብራርተውልኛል፡፡ ስለሆነም ለጥናቱ የሚሆን ናሙና የደም ናሙና ከሆነ ከ7-10 ሚሊ ሊተር ደም እንዲሁም ከህብለሠረሠር የሚቀዳ ፈሳሽ ከሆነ 01 ሚሊ ሊተር ቢወሰድ ተቃውሞ እንዳሌለኝ እና ናሙናውን ለመስጠት ልተባበር ሙሉ ፈቃደኛ መሆኔን ገልጫለሁ፡፡ ከምርምሩ መሳተፍ ወይም አለመሳተፍ ሙበቴ የተጠበቀ መሆኑን እና ላለመሳተፍ ብውስን በሆስፒታል በሚደረግልኝ ሕክምና ላይ ምንም ተፅእኖ እንደሌለው ተረድቻለሁ፡፡

ስለዚህ የጥናቱን ጠቃሚነት ተረድቼ አምኜበት በጥናቱ ለመሳተፍ ፈቃደኛ ነኝ፡፡ በዚህም መሰረት በፈቃዴ መስማማቴን ከዚህ በታች ስሜን በመፃፍ ወይም በፊርማዬ አረጋግጣለሁ፡፡

የህፃኑ ስም ወይም ፊርማ ----------

የምስክር ስምና ፊርማ ----------

Annex-VI-B: English version

Clients ID_____
Name of the child _____
Name of the family/guardian _____ Relationship to the child _____

I am informed about the study titled "Epidemiology, Molecular characteristics and antimicrobial susceptibility pattern of ESBL producing gram negative bacteria among patients visiting JUTH". The objective and application of the study were explained to me. I am informed that all information contained within the questionnaire is to be kept confidential. I am informed about my right. If I don't want to participate, none of my actions will have any problem at all on a service and care I should have. I agreed to give the written assent voluntarily to the researcher to give a specimen (If the specimen is blood 07-10ml of blood and if the specimen is CSF of CSF) for investigation.

I _____ the undersigned hereby give my assent for giving the requested information and specimen for the purpose of the study.

Signature of Child: ----------
Child's parent / guardian): ----------
Witness signature----------

Date ------/-------/----

Annex-VI-C: Afaan oromoo Version

Waliigaltee (qorannoo irratti hirmaachuuf)

Koodii _____
Maqaa daa'imaa_____
Maqaa Abbaa/Guddisaa_____
Firooma qaban_____

Ani, daa'imi maqaan koo armaan olitti ibsame qorannoo baakteeriyaa dhukuba fidan faca'ina isaaniitiif dawaan walbaruu isaanii irratti akka hirmaadhu gaafatamee, waa'ee qoranichaas odeefannoo gahaa argadheera. Kanaaf qorannoo kan irratti hirmaachuun iddattoo qorannoo kanaaf barbaachisu (dhiiga yoo miliilitra 07-10, yoo dhangala'aa lafee keessaa ta'e miliilitra 01) akkan kennu gaafatamee fedha koo ta'uu ibseera.

Qorannoo kana irratti hirmaachuu ykn dhiisuun mirga koo akka ta'e baree, yoon hirmaachuu dhiise tajaajila hospitaala kana keessaa argadhus ta'e, kan biro kamiyyuu irratti dhiibbaa akka hin qabne hubadheera.

Waa'ee qorannoo kanaa ibsa argadheera. Ibsa argadhe kana irraatti hundaa'ee qorannoo kana irratti hirmaachuuf murteeseera. Kanaaf shakkii tokko malee hubannaa kootiin, irraatti walii galuu koo mallattoo koon nan mirkaneessa.

Daa'imaa: Maqaa _____ mallattoo _____
Warra/guddisaa: Maqaa _____mallattoo_____

Maqaa fi Mallattoo Ragaa: _____

ANNEX-VII: Questionnaire

Annex-VII-A: Engish version

Date _____
Code_____

ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCES
DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY

This data collection format is prepared to collect data for a study to be conducted at JUTH among inpatients entitled "The Epidemiology, molecular characteristics and antimicrobial susceptibility pattern of ESBL producing *Enterobacteriaceae* and non-fermentative Gram-negative bacilli at JUTH".

Part I: Demographic data

1.1. Name (optional):

1.2. Card Number:

1.3. Age:

1.4. Sex: Male emale

1.5. Woreda: H

Part II: Clinical data

2.1. Condition of the patient a. Admitted patient utpatient

2.2. Ward admitted to:

i. Surgical:
ii. Medical:
iii. Pediatric
iv. ICU
v. Medical Reason for admission (diagnosis) :

Part three: Antibiotic usage

3.1.Current antibiotic administered (if any)

1. 2. 3.

3.2. Previous antibiotic use:

1. 2. 3.

3.3.Previous admission to this hospital, Yes ☐ No ☐

3.4.If yes to question number 4c state, the date: ☐

3.5.Diagnosis of previous admission: ☐

3.6.In dLength of hospital stay in the previuos admission (specify in days) ☐

Annex-VII-B: Amharic version

አዲስ አባባ ዩኒቨርሲቲ
የጤና ሳይንስ ኮሌጅ
ማይክሮዮሎጂ ኢሚዩኖሎጂ ፓራሳቶሎጂ ት/ት ክፍል

ይህ መረጃ መሰብሰቢያ ቅፅ የተዘጋጀው በጅማ ዩኒቨርሲቲ ሆስፒታል በሚታከሙ ህመሞን ላይ ስለ በሽታ አምጪ ተህዋስያን ስርጭትና መድሃኒት መላመድ ባህሪ የሚደረግ የዘረመል ጥናት መረጃ ለመሰብሰብ ነው፡፡

1. **ክፍል አንድ፡ የግል** መረጃ

1.1.ስም (አማራጭ) ፡

የካርድ ቁጥር ፡

1.2.እድሜ

1.3.ፆታ፡ ወንድ ፡

1.4.ወረዳ ፡ የቤት

2. **ክፍል ሁለት፡ የሕክምና ሁኔታ**

2.1.ተኝቶ የሚታከም ፡

2.2.ተኝተው ከሆነ የተኙበት ዋርድ

I. የቀዶ ጥገና ክፍል
II. የዉስጥ ደዌ ክፍል
III. የሕፃናት ክፍል
IV. ፅኑ ህመማን ክፍል
V. የተኙበት በሽታ ዓይነት ይገለፅ

3. **ክፍል ሶስት፡ የመድሃኒት አጠቃቀም**

3.1.በአሁኑ ሰዓት የሚወሰዱት መድሃኒት ካለ፡

3.2.ከዚህ ቀድም የወሰዱት መድሃኒት ካለ፡

3.3.ከዚህ በፊት ሆስፒታል መጥተው ወይም ተኝተው ያውቃሉ፡ አዎን ☐ ም ፡ ☐

3.4.አዎን ከሆነ መቼ፡ ቀን ይጠቀስ ፡ ☐

3.5.ሆስፒታል የተኙበት በሽታ ምን ነበር ☐

3.6.በዚህ ሆስፒታል ለምን ያህል ጊዜ ተኝተው ታከሙ (በቀን ይጠቀስ) ☐

Annex-VII-C:Afaan oromoo version

YUNIVERSIITII ADDIS ABABA
KOLLEEJJII SAAYINSII FAYYAA
MUUMMEE BARNOOTA MAAYIKIROBAYOLOJII IMUYUNOLOJII, FI PARASAYITOLOJII

Unka: unki ragaa sassaabuuf qophaa'e kun qorannoo hospitaala yuniversiitii jimmaa tti waa'ee baakteeriyaa dhukkuba fiduu danda'anii gosa isaanii, faca'ina isaanii fi amala dawaan wal-baruu isaanii sadarkaa 'DNA' tti qorachuuf gaggeefamuuf gargaara**.**

Kutaa-I: Ragaa dhunfaa

1.1.Maqaa (yoo barbaachise)

1.2.Lakk. Kaardii:

1.3.Saala: Dhi D

1.4.Aanaa: . Manaa:

Kutaa II: Ragaa haala dhukkuba

2. **Haala wal'aansaa**

2.1. Wal'aansa deddeebii: aansa ciisanii:

2.2. Yoo ciisanii ta'e, Eessa:

a) Kutaa baqaqsanii suphuu
b) Kutaa dhukkuba keessoo
c) Kutaa daa'imanii
d) Kutaa knuunsa cimaa
e) Dhukkuba amma ittiin ciiftani

3. **Haala itti fayadama qorichaa:**

3.1. Qoricha amm fudhachaa jiran:

3.2. Qoricha armaan dura fudhachaa turan:

3.3. Kana dura mana yaalaa kana ciisanii turanii: Eeyyee l

3.4. Yoo deebiin 3.3 eyyee ta'e, yoom? Guyaan isaa haa bareefamu

3.5. Yeroo sana dhukubi ittiin ciiftan maal ture

3.6. Yeroo hagam ciiftanii turtan (guyyaa dhaan haaibsamu)

ANNEX-VIII: Laboratory data collection format

ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCES
DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY

This laboratory data collection format is designed to collect laboratory data for the study tilled "Epidemiology, molecular characteristics and antimicrobial susceptibility pattern of extended spectrum β-lactamase producing gram negative bacilli at Jimma University Teaching Hospital".

1. **Specimen type**:

1.1. Blood ☐
1.2. Sputum ☐
1.3. Urine ☐
1.4. Stool ☐
1.5.CSF ☐
1.6. Wound ☐
1.7. Specimen ID: ☐
1.8. Date/Time of collection: ☐
1.9.Culture result

1.9.1. Positive ☐
1.9.2. Negative ☐
1.9.3. Species identified

a. ☐
b. ☐
c. ☐

2. **ESBL screening data**:

2.1. Cefotaxime (30µg): Resistant ☐ Sensitive: ☐

2.2. Ceftazidime (30µg): Resistant ☐ Sensitive: ☐

2.3. Ceftriaxone (30µg): Resistant ☐ Sensitive: ☐

3. **ESBL confirmatory result**

3.1. Cefotaxime +Clavulanate(30µg/10µg): ☐ and cefotaxime: ☐

3.2. Ceftazidime + Clavulanate (30µg/10µg): ☐ and ceftazidime: ☐

4. **Molecular characterization data:**

4.1. Molecular types of ESBL

4.2. Number of ESBL genes

4.3. Resistance genes to other class of antimicrobials

4.4. Virulence genes

4.5. Clonal type

`

Glossary

A-B	
Antimicrobial persistence	Antimicrobial persistence is a phenomenon in which a sub-population of microorganisms is able to survive antimicrobial treatment without acquiring resistance-conferring genetic changes.
Antimicrobial resistance	Antimicrobial resistance is the ability of microbes to grow in the presence of a chemical (drug) that would normally kill them or limit their growth.
C-D	
cgMLST	CgMLST is another tool for typing of bacteria based on core genes
Clonal complex	A group of bacterial isolates showing a high degree of similarity, ideally based on near-identity of multi-locus enzyme profiles and multi-locus sequence types. Clonal complexes are identical to clonal groups.
Clones	Bacterial strains probably arise from a single multiplying bacterial cell, and in this case strains that are similar at least in the seven housekeeping genes (MLST)
Cluster analysis	Comparative analysis of typing data collected for a variety of bacterial isolates in order to group the organisms according to their similarity in these data
Clusters	Bacterial species of certain degree of genetic relatedness
Clusters	The partitioning of a dataset into subsets to reveal groups that share common trait both phenotypic and genetic traits.
E-G	
Epidemic clones	A clone that is suddenly present in a given setting with an unexpectedly high incidence. (However, it is sometimes difficult to determine whether increased incidence is due to strain traits, since there may well be other explanations, e.g., poor hygienic conditions.
Epidemic	The occurrence of an organism above the usual endemic level as evidenced by a larger than expected number of infections. Used as an adjective, the rapid and extensive spread by infection and/or colonization that are widely prevalent, i.e., affecting many individuals in an area or a population at the same time
Extensive drug resistant	Non-susceptible to ≥1 agent in all but ≤2 categories
H-K	
High-risk clones	High-risk clones are globally distributed clones associated with various antimicrobial resistance determinants, ease of transmission, persistence in hosts, and effective transmission between hosts.
Hypermucoviscousity	It is defined by a viscous filament stretching ≥ 5mm when the colony of a bacteria is touched by with inoculating loop. A phenotypic property of usually K. pneumoniae commonly called string test.
Hypervirulence	Hypervirulence is characterized by producing an invasive infection among healthy or immunocompetent person.

L-O

Lineages	Group of isolates sharing essential characteristics due to common descent.
MLST/ST	This is typing or defining bacteria by a types of the house keeping genes (seven genes)
Multi drug resistant	Non-susceptible to ≥1 agent in ≥3 antimicrobial categories.
Mutation	The simplest mutation (change) in a DNA or RNA sequence is a point mutation (a nucleotide change); other mutations include deletion or insertion of one or more nucleotides
Niche	A unique environment or set of ecological conditions in which a specific (micro)organism occurs and thrives.
Outbreak	Local, initially small-scale, cluster of disease generally caused by increased frequency of infection in a distinct population (may be caused by single epidemic strains or combinations of different strains

P-S

Pandemic clone	An epidemic clone that is present in different parts of the world.
Pan-drug resistant	Non-susceptible to all antimicrobial agents that can be prescribed
Pathogenicity	Biological ability to cause disease
Phylogeny	Evolutionary relationships among members of the same taxon (species, strains, etc.).
Population	A group of organisms of the same species inhabiting a given environment
Population dynamics:	The study of factors affecting the variability of populations of microorganisms over time and space, including the interactions of these factors
Population genetics	The study of variation in genes among a group of individual bacterial strains, including the genetic evolution of populations
Population genetics	The study of variation in genes among a group of individual bacterial strains, including the genetic evolution of populations.
rMLST	This a type of MLST based on conserved ribosomal genes
Selection	A natural process resulting in the evolution of an organism that is best adapted to a selective environment
Sporadic	Rare, occurring at un patterned irregular moments and localities, disconnected in space and time; the opposite of epidemic and endemic.
Strain	The descendants of a single isolation in pure culture, usually derived from a single initial colony on a solid growth medium
Successful clones	Clones that can persist a selective environment and form a stable platform for propagation of amicrobial resistance genetic determinants

T-Z

Typing	Phenotypic and/or genetic analysis of bacterial isolates, below the species/subspecies level, performed in order to generate strain/clone-specific fingerprints or datasets that can be used
Virulence	The property of an infectious agent that determines the extent to which an overt disease is produced in an infected population.
Virulence factors	Virulence factors are factors coded by bacterial genome and that enable bacteria to replicate and disseminate within the host in part by subverting or eluding the host defense system
wgMLST	wgMLST is a tool for typing bacteria based on whole genes coded by the bacteria including the core genes and the accessory genes.