Addis Ababa University

College of Health Sciences

Department of Medical Laboratory Sciences

Occurrence and Anti-microbial Susceptibility Pattern of Extended Spectrum Beta-Lactamase Producing Enterobacteriaceae in Governmental Hospital Wastewater in Addis Ababa, Ethiopia

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A Research Thesis Submitted to the Department of Medical Laboratory Sciences, College of Health Sciences, and Addis Ababa University, in Partial Fulfillment of Master of Science Degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology)

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June. 2021G.C

Addis Ababa, Ethiopia

Addis Ababa University

College of Health Sciences
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This is to certify that the thesis prepared by Alehegn Amare Kebede, entitled: **Occurrence and Anti-microbial Susceptibility Pattern of Extended Spectrum Beta-Lactamase Producing ENTEROBACTERIACE in Governmental Hospital Wastewater of Addis Ababa, Ethiopia** and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology Specialty) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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June, 2021 G.C

Addis Ababa, Ethiopia

Acknowledgment:

First of all I would like to thank my Almighty God, St. Virgin Mary's son, for giving me the strength and mercy to pass the challenges I face in this world. I would also like to extend my sincere thanks to my advisors, Mr. Kassu Desta for giving me his scientific guidance and constructive comment during title selection and preparing this thesis.

My sincere gratitude and thanks also goes to Mr. Dejenie Shiferaw, Mr. Tesfaye Legesse, Mr. Adane Amera and Mr.Degefu Beyene for them close moral support, follow up, unreserved scientific and constructive comment during title selection, proposal and this thesis preparation.

I am very grateful to thank Ethiopian public health institution (EPHI) and all microbiology laboratory staffs for giving me permission to conduct this thesis using the institution reusable equipment and by providing reagents and supplies from them in clinical bacteriology laboratories in collaboration with public health microbiology while other costs are covered by the principal investigator.

Lastly but not lest my thank giving also goes to the five hospital medical director, infection prevention and control unit, and building repair and maintenance unit focal person for their loyal cooperative permission to conduct this research in their health institution as well as for their unreserved help without being bored off to show/indicate the sampling unit and its source accurately.

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Abbreviations:

ALERT	All African leprosy and tuberculosis rehabilitation training center,
AMR	Anti-Microbial Resistance
ATCC	American Type Culture Collection
CDT	combination disk Test method
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standard Institute
CTX-M	Cefotaximases Munichin
DDST	Double Disk synergy Test
ESBL	Extended Spectrum Beta-Lactamase
ESBLs-pE	ESBLs producing Enterobacteriaceae
ICU	Intensive Care Unit
MDR TB Ward	Multidrug resistant tuberculosis ward
MDR	Multi-drug Resistance
MHA	Mueller Hinton Agar
MIIRH	Menilik II referral hospital,
SHV	SulfHydryl Variable
SPHHMMC	St. Paul's Hospital Millennium Medical College
TASH	Tikur Ambesa Specialized Hospital
TEM	Temoneira
UTIs	Urinary Tract Infections
WWTP	Wastewater Treatment Plant
Y12HMC	Yekatit 12 hospital medical college

Abstracts

Background: Worldwide, come out and dissemination of Extended-spectrum beta-lactamases (ESBLs) producing Enterobacteriaceae has been warning the efficacy of antibiotics to treat an infection. Hospital wastewater might be a reservoir of such kind of resistant bacteria. Currently, the predominant antibiotics used for the treatment of hospitalized patients infected by Gram negative bacteria are the β-lactam antibiotics. So it is an important source to investigate the magnitude of ESBLs producing bacteria and their antimicrobial susceptibility pattern.

Objective:-To determine the occurrence of ESBLs producing Enterobacteriaceae(ESBLs-pE). and their antibiotic susceptibility pattern in wastewater released from governmental hospitals in Addis Ababa, Ethiopia.

Method: Across-sectional study was carried out from April 1 to May 31, 2020. A total of 100 wastewaters were collected from five governmental hospitals in Addis Ababa using a grap-sampling technique. All Enterobacteriaceae were screened for ESBLs production using cefotaxime and ceftazidime as per 29th CLSI guideline. Each screen positive for ESBLs production was confirmed by the combination disk method (CDT) and their antibiotic susceptibility pattern were done using the Kirby-Bauer disk diffusion method on Muller Hinton agar (MHA). Data were entered and summarized using SPSS version 20 software.

Results: Of all Enterobacteriaceae, 48.3% were confirmed ESBLs-pE. The highest ratio of ESBLs-Pe was observed in adult ward (66.7%) and laundry unit effluent (58.8%). The highest ESBL producers were *E. coli* (21.8%) and *K. pneumoniae* (4.8%). The most elevated resistance level of ESBL producer were observed to cefotaxime (95.8%) and amoxacilline/clavunalate (93%). 64% of tested Enterobacteriaceae isolates were multi drug resistant (MDR).

Conclusion: Higher magnitude of MDR and ESBLs-pE were present in the hospital wastewater. Majority of them were in adult ward and laundry unit effluents. The most frequent ESBLs-pE was among *E .coli* and *K. pneumoniae*. Hence, Consistent infection prevention and control procedures should be in practice at each ward/unit.

Keywords:-Hospital, Wastewater, Beta Lactamase, ESBLs, Enterobacteriacea, Multi-drug resistance

1. Introduction

1.1.Background

Wastewater refers to water used by humans and loses its quality and usefulness that embrace waste liquid of domestic, agricultural, commercial sources, industries, and hospital sources. Every these anthropogenic activity generates wastewater as a result of physical, chemical and biological quality of water deterioration (1). The effluent from hospital contains a lot of drug-resistant pathogens, a larger form of chemicals, solvents, disinfectants and a lot of risky materials like pharmaceuticals and radionuclides than domestic sewerage (2). As a result of this hospital effluent contains antibiotic residues that are enough to kill susceptible bacteria and at the same time increases the number of resistant bacteria (3). The presence of antibiotic-resistant microorganism in effluent and sewage system line is a growing public health concern (4). Since one of the main ways of diffusion of pathogenic and/or antibiotic resistant microorganism is thru water, soil and air. This phenomenon results multi-drug resistant (MDR) microorganisms that have been revealed in different water sources including rivers, lakes, groundwater and drinking water (5-8). The discharge of resistant bacteria to the receiving aquatic environment will create public health impact through, carrying transmissible gene, by acting as a vector or reservoir of resistant gene (9, 10).

The members of the Enterobacteriaceae are gram-negative bacilli, which are usually resident in the gastrointestinal tract. Instance of such organisms consisting of *E. coli*, *K. pneumoniae*, *Enterobacter cloacae, Citrobacter freundii* and *Proteus mirabilis.* In patients hospitalized in intensive care units (ICUs), the Enterobacteriaceae holds for about one third of all cases of ICU-acquired pneumonia, one third of all cases of ICU-acquired urinary tract infection, and 10% to 15% of ICU-acquired bloodstream infections (11).

Currently, the dominant antibiotics used for treating hospitalized patients infected by Gram negative bacteria are the β-lactam antibiotics that inhibit transpeptidases taking part in bacterial cell wall synthesis. Sadly, these beta-lactam antibiotics will be deactivated by β-lactamase enzymes (12). A persistent exposure of bacterial strains to a multitude of beta-lactam antibiotics has evoked a dynamic, continuous production and mutation of beta lactamase in the

bacteria resulting in the event of extended spectrum beta-lactamases (ESBLs) inflicting resistant to broad spectrum beta-lactam antibiotics (12-15).

ESBLs are beta-lactamases that confer resistant to the penicillins; first, second, and third generation cephalosporins and monobactams by hydrolysis of these antimicrobials. Additionally, these enzymes are pent-up by beta-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. ESBLs are encoded on transferrable conjugative plasmids that facilitate widespread dissemination, not solely between the identical species of bacteria, but also across completely different species. Furthermore, these plasmids code for resistant to other classes of potent antimicrobial agents, in significantly, aminoglycosides and fluoroquinolones (16).Among the numerous ESBLs described in a variety of pathogens, CTX-M, TEM, and SHV types proved to be the most successful in terms of promiscuity and dissemination across various epidemiological niches (17).

The first ESBLs were isolated in the 1980's (18, 19). ESBLs are isolated from a large type of Enterobacteriaceae (foremost in *E. coli and K. pneumoniae),Pseudomonas aeruginosa* and *Capnocytophaga ochracea (20).* The increasing prevalence of ESBL Entrobacteriaceae esp. *K. pneumoniae* and *E. coli* worldwide is a source of explicit concern. The hospital sewage or the low effectivity of hospital waste -treatment plants may lead to the dissemination of ESBL-producing bacteria. The permanent presence of detectable antimicrobial levels within the hospital wastewater treatment may powerfully influence the environment, creating a selective pressure that would be ultimately responsible for the dominance of resistant microorganisms among those present in the habitat.

In the clinical microbiology laboratory, ESBL producing Gram-negative bacteria are outlined by (i) reduced susceptibility to one or more of the indicator cephalosporin antibiotics (ceftazidime, cefotaxime, ceftriaxone, cefpodoxime or aztreonam), and (ii) synergism of the activity of those indicator antibiotics within the presence of a β-lactamase inhibitor like clavulanic acid (21).

1.2. Statement of the Problem

Hospital wastewater is venturesome to public health and ecological balance (22). Several non-metabolized medication excreted from patients enter into wastewater, that finally interacts with the microflora of hospital waste matter. These microflora include saprophytic bacteria from the atmosphere, medical devices, soil, and water used in the hospital practice and the pathogens which are mainly released with patient excreta (23). As much as 50-90 % of an administered drugs are excreted by the body in a biologically active form (24), this makes antibiotics to exist in hospital wastewater, for instance some sulfonamides and fluoroquinolones(25). In hospital wastewater collecting tank/manhole antibiotic residues can favor the development of antibiotic resistant bacteria because of the selective pressure (by kill susceptible bacteria) placed on bacteria(26, 27).

The emergence of multi-resistant in the Enterobacteriaceae family is an inquire that requires attention, because these are useful causative agents of hospital infections, basically related with pneumonias, urinary tract infections, bacteremia, blood stream infections, and other intra-abdominal infections(28, 29). Anti-microbial resistant (AMR) is the predominant issue causing a threat to public health, with many reports warning of the significant risk of a post-antimicrobial era wherein common infections can kill (30, 31). In recent time, resistant infections result in 700,000 deaths every year, but the global resistance-associated total cost & death is estimated to be ≈100 trillion USD & 10 million lives per year in 2050 respectively (32, 33). Meanwhile the European Center for Disease Prevention and Control and the US Centers for Disease Control and Prevention have reported that AMR infections resulted in 25,000 and 23,000 deaths every year in high-income countries in Europe and the USA, respectively. In low and middle-income countries, AMR infections were accountable for the deaths of 58,000 children's and 38,000 adults in Thailand and India correspondingly (34).

Recently, the World Health Organization (WHO) Global Antimicrobial Surveillance System (GLASS) identified & reported 12 bacterial species which shows increased levels of resistant in many regions of the world that pose the greatest threat to human health (35). This list specifically consisting of Gram-negative bacteria and the most frequent etiologic agents related with hospital and/or community-acquired infections. These AMR bacteria had been divided into three categories: critical, high and medium priority, based on their effect on human health and

the urgency for the innovation of new antimicrobial drugs to treat resistant infections. The critical category consisting of *P. aeruginosa* (carbapenem-resistant), *Acinetobacter baumannii* (carbapenem-resistant) and various Enterobacteriaceae members, comprising *E. coli*, *Klebsiella* spp., *Serratia* spp., and *Proteus* spp. (carbapenem-resistant and ESBL-producing), which are related with severe and, often deadly, infections, consisting of pneumonia and bloodstream infections (36).

Globally, β-lactam antibiotics are widely used for the treatment of pneumonia, urinary tract and bloodstream Gram negative bacterial infections. It is more or less 50% of all prescribed antimicrobial belong to this group (17, 37). However, β-lactamases enzymes which are produced by microorganisms hydrolysis β-lactam rings to inactivate them (17). ESBLs are mutant forms of β-lactamases enzymes coded by gens located on transferable plasmids, which might simply spread from one organism to a different. The ESBLs producing organisms are typically MDR, because the plasmids producing ESBLs may carry resistant to other antibiotics. Drug resistant of this kind is commonly tough to recognize by using conventional antimicrobial susceptibility methods. Failure to identify ESBL producing bacteria conjointly contributes to their undetected spread. Therefore, identification of the resistant phenotypes is vital, in developing countries where there's excessive use of antibiotics and lack of adequate antimicrobial resistant surveillance (38).

Infections caused by ESBLs producing organisms have been described as an emerging public health problem worldwide. Because of their extraordinarily difficult to treat and increased mortality related to them (16). Patients infected with ESBLs producers experience greater probability of poor treatment outcome with mortality rates ranging from 42-100% (39). The ESBL producing Enterobacteriaceae(ESBLs-pE) are becoming increasingly prevalent not only in hospital environments but also in water especially wastewater (40), soil, and food such as fresh vegetables and meat. A study conducted in Gondar, Ethiopia reported that 14.8% ESBLs-pE from hospital environments of that the predominant isolates were *K. pneumoniae* (14.7%) and *E. coli* (12.3%) (41). Another studies in Bahir Dar, Ethiopia reported that the overall prevalence of ESBLs-pE in drinking water samples were 9.4% (42).

In developing countries like Ethiopia, wastewater generated from most hospitals is directly discharged to the nearby streams and rivers without getting appropriate treatment before

being released. In Sub-Saharan Africa, 50 to 90% of the vegetables consumed by city dwellers are cultivated in urban or peri-urban areas mainly with wastewater (43). Here in Ethiopia also contaminated rivers are customary used for irrigation purpose by urban farmers to cultivate vegetables without any precaution. This may serve as source of infection with MDR and ESBLs producer pathogenic bacteria to the communities (44).

In Ethiopia, specially, in Addis Ababa, there's limited data regarding resistant pattern of microorganisms recovered from hospital effluent. This study is therefore designed to provide insight in the antimicrobial susceptibility profile and occurrence of ESBLs-pE in different hospitals of Addis Ababa wastewater. I become fascinating to check this as a result of beta lactam antibiotics are the key antibiotic classes in the anti-infective therapy and current preponderantly existence of ESBLs-pE especially among *K. pneumoniae* and *E. coli*.

1.3.Significance of the Study

The study provided information about the occurrence and magnitude difference of ESBL producing Enterobacteriaceae from different hospital effluent.

The study availed baseline information on antimicrobial resistance pattern for Enterobacteriaceae isolates recovered in hospital wastewater with their multidrug-resistance level for infection prevention and control.

The study generated important finding that can be used by different responsible bodies such as hospital infection prevention and safety committee, minister of health and others police makers.

2. Literature review

2.1.Hospital Waste Water

In hospital, water consumed by totally different parts like in several admission ward, laboratory and pharmacy room, administrative unit, laundry section, health professional residential apartment, hospital café and restaurant service provider and others. Each of these phylogeny activity generates wastewater as a result of physical, chemical and biological quality of water deteriorated (1). The volume of wastewater produced in health-care facilities like hospitals is best measured by water consumption. Water consumption by hospitals depends heavily on factors like the type of healthcare services provided, range of beds, accessibility to water, climatic situation, level of care and native water-use practices (45). Relying upon such factors, the quantity of wastewater released from hospitals may vary from hospital to hospital however it's been estimated as 400 to 1200 liters/bed/day. For instance, per capital production of wastewater per bed per day in America is 1000 liters (2).

Hospital wastewater generates risk waste like infectious, pathological, sharps, pharmaceutical, genotoxic, chemical, and radioactive wastes. Hence, study conducted on bacteriological and physiochemical qualities of hospital wastewater unconcealed that there was contamination of the receiving environment (water, soil and air) thanks to the discharge of hospital wastewater that might be dangerous to human and animal health (46).

2.2.Enterobacteriaceae

Antibiotic resistant is a main public-health problem globally wherein hospitals and hospital effluents act as a hotspot for antimicrobial-resistant bacteria. The dissemination of MDR bacteria via hospital wastewater is an important cause for consideration since it is believable that MDR bacteria are selected mainly in hospitals and discharged into wastewater. Enhanced antibiotic consumption and disposal is causing to higher levels of bacterial resistance (4).

A study conducted in 2008 in Rio de Janeiro, Brazil, from hospital wastewater 44 % of isolated bacteria were ESBL producer using double disk diffusion method. Distributions of the ESBL-producing isolates from influent, clarifier tank effluent and chlorine contact tank effluent were 39 %, 46 % and 56 % respectively. In that study, the most common ESBL producing

isolates was *K. pneumoniae* and *E. cloacae* in wastewater samples. Again this study, observed higher antimicrobial resistant rates to amikacin and trimethoprim／sulphametoxazole among the non-beta-lactam antibiotics, while among antibiotics of the beta-lactam group, cefalothin showed the highest resistant rate (47).

Other study in the same year 2008 in Goiânia, Brazil from sewage effluents of 10 hospitals and sewage treatment station of the city 67 microorganisms were isolated and identified, including *E. coli* 10 (14.92 %), *K. pneumoniae* 10 (14.92 %), *P. aeruginosa* 3 (4.47 %) and *A. baumannii* 1 (1.49 %). Of the *E. coli* strains, 100 % were resistant to aztreonam, 40 % to ampicillin, 30 % to piperacillin, 20 % to ciprofloxacin and 10 % to gentamicin. None of the bacterial strains produced ESBL using double-disk diffusion test. Strains of *K. pneumoniae* were resistant to ampicillin (70 %) and to piperacillin (20 %); additionally, 50 % showed intermediate resistant to piperacillin. Overall, resistant rates were less within the isolates of *E. coli, P. aeruginosa, K. pneumoniae* and *A. baumannii*(48).

In 2014-2015 a study in Italy from well, river water and wastewater treatment plant (WWTP) identified 22.7 % Enterobacteriaceae showing a synergic effect by double disk phenotypic test using third generation cephalosporin and piperacillin-tazobactam; from this, 50 % was *E.coli* (49). In the same year (2014-2015) a study carried out in Spain reported the average counts of ESBLs-pE in hospital sewage were more elevated (more than 2.5 logs) than those noticed in urban wastewater; even though, the total level of Enterobacteria difference among the urban and hospital sewage was little (50).

A two years (2017-2018) study conducted from three hospitals wastewater of Slovenian (one hospital) and Austrian (two hospital) revealed ESBLs-pE in the range up to 10^7 CFU/ml which was similar to previously published results by Morris *et al*, 2016. In that study the highest concentration of ESBLs-pE according to ChomID ESBL agar identification was present in wastewater from the Slovenian hospital than Austrian hospital but there were a slight significantly difference in concentration of ESBL producing bacteria between 2017 and 2018 in the Austria hospital but not in Slovenian general hospital (51).

In Bangladesh, a study titled as ESBLs-pE in Aquatic Environmental Sources was carried out in the year 2012-2014 and reported 29 % isolates were ESBL producer from the 44 MDR

isolates according to the double disk synergy test (DDST) method. In this study the most common resistant phenotype of the water isolates was to amoxicillin and aztreonam(52).

Other laboratory study in Bangladesh published in 2014 from two hospital wastewater recovered a total of 104 bacterial Isolates, among this 32 (30.7%) were *E. coli* and *Klebsiella* spp., 26 (25%) were *Enterobacter* spp., 8 (7.7%) were *Proteus vulgaris* and 6 (5.8%) were *Salmonella* spp., respectively. In this study, regardless of bacterial type, the highest percentage of resistant was shown against Amoxicillin and Ampicillin where 78 (75%) and 66 (63.5%) isolates were found to be resistant respectively. On the other hand, ciprofloxacin showed less resistant pattern in contrast to amoxicillin and ampicillin (53).

A study conducted in 2018 in Nepal from seven hospital wastewater collected at different location found out of 23 isolates, 69.6% were MDR and 30.4% were ESBLs-pE using combined disk test (CDT) method. In this study, the percentage of ESBLs-pE was 60%, 40% and 25% for *Enterobacter* spp., *Citrobacter* spp. and *E. coli* respectively. Meanwhile, the bacterial distribution in 23 isolates was *E. coli* (34.7%), *Citrobacter* (21.7%), *Enterobacter* (21.7%), *Klebsiella* (13%), *Shigella* (4.3%) and *Yersinia* (4.3%). Again in this study, most of the bacteria isolated were resistant to ampicillin, ceftazidime, cefpodoxime, amoxicillin/clavulanate, cefotaxime, cefoxitin, ceftriaxone, and cefuroxime. The isolates were sensitive to nitrofurantoin, azithromycin, trimethoprim/sulfamethoxazole, chloramphenicol, ciprofloxacin, ofloxacin, and gentamycin antibiotics (54).

In 2019 study conducted in China from hospital sewage, treated effluents and receiving rivers revealed a total of 62 (59.6%) out of the 104 isolates were found to be Enterobacteriaceae; from this, the predominant isolates were *E. coli*, *K. pneumoniae* and *Enterobacter* spp. with a percentage of 56.5%, 8.1%, and 4.8% respectively. In that study, all Enterobacteriaceae strain isolated (62) revealed resistant to ampicillin and cefotaxime. In the same talk, 85.5% Enterobacteriaceae isolates were MDR strain. A total of 91.4% (32/35) *E. coli* and 94.1% (16/17) *K. pneumoniae* isolates were identified to be MDR (55).

According to study done in Dubai, UAE from meat and municipality wastewater published on 2020 reported 41.7% potential ESBLs-pE screened by ESBL chromogenic agar. However; 40.7% (35/86) potential ESBLs-pE was from wastewater. In this study of all the

potential ESBL producing isolates, 22% (19/86) were confirmed as ESBL producers, while 10.5% were from wastewater by a double-disc diffusion test method with the fourth generation cephalosporin–Cefpirome. Again, this study observed all potential 86 ESBL isolates found to be resistant to one or more third-generation cephalosporins, with 93% of the isolates, have shown resistant to cefpodoxime followed by cefotaxime (79%), ceftizoxime (78%), and ceftazidime (70%) (56).

A study carried out in the period 2012 - 2013, in Egypt from a sewage treatment plant of Alexandria city obtained 69.8% and 57.7% ESBLs producing Gram negative bacteria in the influent and effluent sewage respectively using CDT method . In this study, a total of 87 ESBLs producing isolates with *E. coli* (74 isolates) and *K. pneumonia* (13 isolates) were identified. Meanwhile, the bacterial isolates showed highest antimicrobial resistant rates to amoxicillin-clavulanic acid, piperacillin-tazobactam and trimethoprim/sulfamethoxazole (57).

A study conducted on 2015 in South Eastern, Nigeria, from three hospital wastewater effluent recovered 61 bacterial isolates with the most frequently isolated bacteria were *P. aeruginosa* (27.9%) and *E. coli* (26.2%), while the least number of isolate was *Salmonella* spp. (21.3%). In this study, all the bacterial isolates exhibited MDR (resistant to more than three antibiotics), although their patterns of resistant varied (58)**.**

In 2015 other study, in Nigeria from three river water discovered 17.9% (37/207) ESBL producer from beta-lactam resistant Gram negative bacteria using DDST method. In this study, among the ESBL-producers, 35.1% were *K. pneumoniae*, while 91.9%, 73.0% and 64.9% of ESBL isolates showed resistant to cefotaxime, cefepime and aztreonam, respectively. Furthermore, they were also resistant to ciprofloxacin (8.1%) and gentamicin (18.9%) (59).

Only a few researchers were carried out in Ethiopia on the proportion of ESBL in hospital wastewater. In 2012 a study of wastewater from hospital and non-hospital source in Gondar, recovered 113 isolates with the most common identified bacterium was *Klebsiella* spp. (26.6%), *Pseudomonas* spp.(16.8%), *E. coli* (11.5%) and *Citrobacter* spp.(11.5%) respectively. In this study, the overall prevalence of MDR was 69.9% (79/113) (60).

Another cross sectional study conducted on 2014 in Gondar referral hospital environment, depicted 14.8% ESBLs-pE, wherein the frequently isolates were *K. pneumoniae*

(42.10%), *E. coli* (35.1% and *Proteus mirabilis* (7%) correspondingly. In that study, the highest numbers of ESBLs-pE isolates were found from sinks (22.80%), bed tables (22.80%) and wastewater (15.79%) respectively. Again that study, observed all ESBLs-pE has been observed to be 100% resistant to ceftriaxone, cefpirome, ceftazidime, cefpodoxime, and amoxicillin with clavulanic acid. In addition, resistant proportion was elevated for non-beta-lactam antimicrobials such as chloramphenicol (70.18%), cotrimoxazole (64.91%), ciprofloxacin (43.86%), norfloxacin (42.10%), and gentamicin (19.30%) (61)**.**

In 2015 a study from treated and untreated hospital wastewater at Ayder referral hospital, North Ethiopia, detected 134 bacterial isolates, 62.7% from untreated and 37.3% from treated sewage; whereas, *Klebsiella* spp. was the most frequently isolated bacteria from both site. In that study, the overall MDR proportion was 79.1% from the two sample collection site (62).

A study conducted on 2017 in Addis Ababa from two hospital wastewater, abattoir, downstream rivers and a WWTP identified a total of 54 bacterial isolates, from this the most frequently isolates were *E.coli* (32%), *Salmonella* (23%), *K. pneumonia* (15%), and *E. cloacae* (11%) respectively. In this study, all isolates were resistant to two or more antimicrobials tested; whereas, MDR was more commonly observed in *Citrobacter*, *Enterobacter* and *E. coli* isolates and it was more common in isolates obtained from hospital effluent than other sources. ESBLs producer was identified in 27.3% of MDR isolates using DDST method, all of them acquired from hospital effluents (63)**.**

3. Objective

3.1.General Objective

- ✓ To determine the occurrence of ESBL producing Enterobacteriaceae(ESBLs-pE) and anti-microbial susceptibility pattern in wastewater released from selected governmental hospitals of Addis Ababa, Ethiopia.

3.2.Specific Objectives

- ✓ To assess the magnitude of ESBLs-pE from five governmental hospitals effluent at different sampling site.
- ✓ To compare the magnitude of ESBLs-pE among five hospitals wastewater.
- ✓ To determine antimicrobial susceptibility pattern for Enterobacteriaceae isolate in five governmental hospitals wastewater in Addis Ababa, Ethiopia.

4. Hypothesis

The occurrence/magnitude of MDR and ESBL producing Enterobacteriaceae in hospital effluent of Addis Ababa is different from previous study.

5. Materials and methods

5.1.Study Area

This study was done in Addis Ababa which is the Capital City of the Federal Democratic Republic of Ethiopia, the diplomatic capital of Africa and the seat of different international and regional organizations. The city administration is divided in to ten sub city and 116 woreda. It hosts an estimated 3.2-38 million people, which is a 17% share of Ethiopia's total urban population. Presently, Addis Ababa is experiencing an annual growth rate of 3.8% and is speculated to reach 4.7 million residents by 2030. The city covers a Landmass of 540 square kilo meters. The city is located at the heart of the country, at an altitude ranging from 2,100 meters at Akaki in the south to 3,000 meters at Entoto Hill in the North (64).

Within the city there are 13 government hospitals (five federal, six under Addis Ababa health bureau, one owned by police force and one armed force hospital) distributed throughout 10 sub cities. For this study, it was necessary to pick out major hospitals with a lot of instrumentation, a variety of medical services, higher bed and large run of patients. Hence, hospitals having 200 or more beds and those which provide 10 or more type of medical services are considered as major. Seven governmental hospitals in Addis Ababa meet these criteria. Out of these, 5 hospitals are selected randomly to making a sample size of 38.5%. Ten percent or more samples is considered as a good sample size for small populations (65). The selected hospitals included: Tikur Ambesa Specialized Hospital (TASH) having 543 beds and providing 26 different medical service, St. Paul's Hospital Millennium Medical College (SPHMMC) with 337 beds and providing 25 different medical service, All African leprosy and tuberculosis rehabilitation training center (Alert hospital) with 241 beds and providing 16 different medical service, Yekatit 12 hospital medical college (Y12HMC) with 210 beds and 19 different medical service and Menilik II referral hospital (MIIRH) with 203 beds and 15 different medical service(66).

TASH is the largest and oldest specialized teaching hospital which is open 24 hours for emergency services located in Lideta sub city at Addis Ababa. TASH providing referral services for approximately 370,000- 400,000 patients a year for patients coming from all side of the

country with 130 specialists, 50 non-teaching doctors and other professionals. The emergency department visited by around 80,000 patients a year. The hospital provides service to outpatients and patients admitted in different wards such as medical, surgical, pediatric, neonatal, obstetric and gynecologic.

St. Paul's Hospital Millennium Medical College, the second largest public hospital in Ethiopia which is located in Gulele sub city at Addis Ababa. An estimated 800 clinical and non-clinical staff providing care to approximately 110,000 people each year. St. Paul's receives referrals from around the country and providing inpatient and specialized procedural services, Screening & Diagnostic Services like Radiology, Pathology and Laboratory service and others.

Laboratory analysis was conducted at Ethiopian public health institute (EPHI) in clinical bacteriology and mycology national referral laboratory in collaboration with food microbiology laboratory. The laboratory has been accredited by Ethiopian National Accreditation Office.

5.2.Study Design and Study Period

A cross sectional study design was employed from April 1 to May 31, 2020.

5.3.Sample

5.3.1. Source Sample

All wastewater collection manholes at thirteen governmental hospitals (n=13) in Addis-Ababa were taken as source sample.

5.3.2. Study Sample

All wastewater collecting manholes at five governmental hospitals (n=5) in Addis-Ababa were taken as study sample.

5.4.Inclusion and Exclusion Criteria

5.4.1. Inclusion Criteria

Governmental Hospitals which having 200 or more beds, and providing 10 or more type of medical services and volunteers to participate in this study were included.

5.4.2. Exclusion Criteria

Governmental hospitals which haven't wastewater collecting manholes and difficult to segregate hospitals' effluent origin. In addition, those which provide medical service of non-infectious disease (e.g. Mental Specialized Hospital).

5.5.Study Variable

5.5.1. Dependent Variable

- Enterobacteriaceae isolates
- ESBL producing Enterobacteriaceae
- Anti-microbial pattern of Enterobacteriaceae isolates

5.5.2. Independent Variable

- Study area/selected hospital
- Sampling site
- Time of sample collection
- Frequency of sampling

5.6.Sampling Frequency and Sampling Technique

Hospitals that have higher bed and serving many patients were selected in ten sub- city of Addis Ababa. Based on these five governmental hospitals were included for this study. In each hospital, six sampling sites are employed to collect hospital effluent. These are a manhole used to collect a wastewater originate from adult ward, pediatric ward, labor ward, laboratory unit, laundry unit and a manhole of septic tank (which hold effluent from all source and we termed Mixed). In addition, one sampling unit viz. MDR TB ward was incorporated to collect hospital effluent only in alert hospital.

A total of 100 discrete (that is 50 at the morning and 50 at the afternoon) wastewater samples were collected from each ward/unit wastewater collecting manholes at the sampling site with four hour interval in the study period. This encompassed discrete effluent of 24 from each Y12HMC and MIIRH, 20 from each SPHMMC and ALERT, and 12 discrete effluents from TASH.

A "Grab sampling technique" was applied to collect the most representative samples according to guidelines of wastewater sampling techniques stated on Environment Protection Authority (EPA)(67) and American Public Health Association (APHA)(68). Discrete samples were collected in two rounds in each hospital for two months. The 1^{st} and 2^{nd} round samples were collected within 15 day interval. In each round, the discrete samples were collected two times a day with four hour interval from each sampling sites in each hospital in 150 ml cleaned and sterile microbiological glass bottles. Here 150 ml sterile glass container was used to collect 125 ml wastewater samples.

Hospitals' effluent Samples were collected during their maximum activity period (usually 10:00 am- 2:30 pm) according to the method used by Nuñez and Moretton (10). In addition, samples were collected near the center of flow channel, at approximately 10-15 centimeter of the water depth, where the turbulence was at maximum and the possibility settling was minimized. Grazing (skimming) the water surface or slogging the bottle was avoided. The first sample was collected in the morning 10 -10:30 AM whereas the second sample was collected at 2-230 PM from each sampling site. After taking the sample, the neck of the bottle was wiped with 95% alcohol then the sample bottle labelled with date, code number, and time and sampling site. All

samples were collected manually and transported instantly to Ethiopia Public Health Institute (EPHI) food microbiology and clinical laboratories with cold chin (4˚C) for bacteriological analysis within six hours of collection. A pair of new, non-powdered, disposable glove, a suitable gown and eye google were used each time while, collecting samples to avoid personal contamination. In the same token, heavy duty glove was used to clean and pick up the cover of manholes at time of sample collection.

5.7.Sampling Site

In present study, there were different wards and units in the selected hospitals. Each ward/unit generated wastewater having different characteristics. So in order to locate in which sampling site the isolate found, the hospital effluents were collected at different manhole of each units/wards of hospital. The hospital effluents were collected at manhole of the adult ward, pediatric ward, delivery ward, laboratory unit, laundry service unit and at sampling site named MIXED (for this study purpose only). Here mixed sample indicates a hospital effluent in which its origin holding from different ward/unit and flow together hence it was difficult to identify at which specific unit/ward it came. In addition, wastewater was collected from MDR TB ward in case of Alert hospital only. The sampling site manholes located just at the outlet of wastewater of each ward/unit before discharged in to receiving water/ collecting septic tank and at the side of each ward/unit building. The geographical position of the sampling site/ unit was obtained and documented (Table 1).

Table 1. Name of hospital and wastewater sampling unit/site with their geographical location, at selected governmental hospitals wastewater, Addis Ababa, Ethiopia from April 1 to May 31, 2020.

Variables		**Geographical location of each hospital sampling units**				
		TASH	**SPHMMC**	**ALERT**	**Y12HMC**	**MIIRH**
Sampling units	**ADULT WARD**	N9^{0}1'11"E38^{0}44'59"	N9^{o}2'50"E38^{o}43'43"	N8^{0}59'8"E38^{0}42'42"	N9^{0}2'33"E38^{0}45'35"	N9^{0}2'15"E38^{0}46'36"
	PEDIATRIC WARD	N9^{0}1'15"E38^{0}44'55"	N9^{0}2'56"E38^{0}43'43"	NC	N9^{0}2'35"E38^{0}45'38"	N9^{0}2'19"E38^{0}46'30"
	LABOR WARD	N9^{0}1'12"E38^{0}44'57"	NC	N8^{0}58'58"E38^{0}42'41"	N9^{0}2'31"E38^{0}45'37"	N9^{0}2'20"E38^{0}46'30"
	LABORATORY UNIT	N9^{0}1'12"E38^{0}44'58"	N9^{0}2'54"E38^{0}43'40"	N8^{0}59'4"E38^{0}42'43"	N9^{0}2'36"E38^{0}45'37"	N9^{0}2'18"E38^{0}46'29"
	LAUNDARY UNIT	N9^{0}1'13"E38^{0}44'59"	N9^{0}2'53"E38^{0}43'42"	N8^{0}59'4"E38^{0}42'44"	N9^{0}2'45"E38^{0}43'51"	N9^{0}2'17"E38^{0}46'36"
	MIXED SAMPLE	N9^{0}1'16"E38^{0}45'55"	N9^{0}2'50"E38^{0}43'42"	NC	N9^{0}2'35"E38^{0}45'42"	N9^{0}2'18"E38^{0}46'26"
	MDR TB WARD	NC	NC	N8^{0}59'7"E38^{0}42'48"	NC	NC

Note: N=north, E=east, NC= sample is not collected, '= Minute, "=Second

TASH=Tikur Ambesa Specialized Hospital, SPHHMMC= St. Paul's Hospital Millennium Medical College, ALERT= All African leprosy and tuberculosis rehabilitation training center, Y12HMC= Yekatit 12 hospital medical college, MIIRH= Menilik II referral hospital, MDR TB Ward = multidrug resistant tuberculosis ward,

N.B. The Accuracy of geographical location obtained using google map GPS coordinator is around 5-10 meters

5.8. Data Collection Procedure

The important information's were recorded using a pre-developed data collection form by asking the authorized body (about sampling unit/site, wastewater disinfection and disposal procedures), from record book/file (e.g. number of patients served during study period) and using google map application (for geographical location of the sample). After the sampling unit/site at each hospitals identified and its location as well as the source of wastewater, aseptically with care, the cover of each manholes are lifted up to collect the wastewater then covered immediately. The name of the hospital and sampling unit, time and round of collection, as well as its geographical location are recorded on pre-developed data collection format in addition to, labeling collecting bottle at time of sample collection. All of this information was collected by the principal investigator.

5.9. Laboratory Analysis

5.9.1. Isolation and Characterization of Pure Cultures

For isolation the bacteria, a loopful of a well-mixed sample suspension was inoculated using sterile inoculating loop on to MacConkey agar pate (Oxoid LTD, Basingstoke, Hampshire, England) and incubated aerobically at 37 ^{o}C for 18-24 hours.

After incubation for 24 hr. at 37^{o}C, bacterial colonies with distinct coloration and morphology were randomly picked up and sub cultured on to another MacConkey Agar plate for further purification. Then purified colonies with distinct presumptive colonies of each suspected bacterial species and fermentation on MacConkey agar are sub cultured on tryptic soya agar (TSA)/nutrient agar (Oxoid LTD, Basingstoke, Hampshire, England) depending on the availability of media for biochemical test.

For identification pure colony from non-selective nutrient agar/TSA was sub cultured and identified based on the following biochemical tests: oxidase, indole, urea, motility, Lysine decarboxylase, citrate utilization and triple sugar iron tests as per the standards of microbiology procedure including *E. coli* ATCC 25922, as a control culture (69). Following purification and species identified, two –three purified colonies were preserved in Skimmy milk at -80^{o}C for further characterization, after each isolate was assigned a unique identification number.

5.9.2. Screening isolates for ESBLs Producing

Those Enterobacteriaceae that was resistant or reduced susceptibility to the screening indicator cephalosporin (cefotaxime and/or ceftazidime) was considered as suspicious of ESBLs production. In other word, isolates that showed an inhibition zone size of ≤27 mm for cefotaxime (30µg) and/or ≤ 22 mm for ceftazidime (30µg) were considered as suspicious ESBL producers and selected for confirmation for ESBLs production.

5.9.3. Confirmation of ESBLs Producing Enterobacteriaceae

Confirmation of suspicious ESBL-producing isolate was verified by the combination disk method (CDT) as delineated by the 29th edition CLSI guide line (70). The test was performed using two cephalosporin antibiotics: ceftazidime (30µg), and cefotaxime (30µg) alone and in combination with beta-lactam inhibitor ((ceftazidime- clavulanic acid (30/10µg), and cefotaxime-clavulanic acid (30/10µg)) by dispensing on 0.5 McFarland turbidity bacterial suspension inoculated Muller Hinton agar (MHA) plate(Oxoid LTD, Basingstoke, Hampshire, England) and then incubated overnight (18-24 hours) at 37 ^{o}C as per 29th edition CLSI guideline. ESBL production was considered positive when ≥ 5mm increase in the zone diameter for the ceftazidime or cefotaxime tested in combination with clavulanic acid versus its zone when tested alone (70). *E. coli* ATCC 25922 was used as a negative control throughout the tests as a non-ESBL culture.

5.9.4. Antimicrobial Susceptibility Testing

Once the bacteria were isolated and identified from each sample collected, all Enterobacteriaceae isolates were assessed for non-susceptible pattern for 12 antibiotic agents by using the Kirby-Bauer disk diffusion method on MHA in line with 29th edition CLSI guideline (70). Bacterial inoculum was prepared by suspending the freshly grown bacteria in 4–5 ml sterile normal saline and the turbidity was adjusted to that of a 0.5 McFarland standard. Then a prepared bacterial inoculum suspension (0.5 McFarland standards) was streaked on MHA using sterile swap applicator stick and antimicrobial discs were placed. The antibiotic discs used for this study were: trimethoprim⁄ sulphametoxazole (SXT, 1.25/3.75ug), ciprofloxacin (CPR, 5ug), tazobactam+piperacillin (TZP, 30ug), cefoxitin (CXT, 30µg), chloramphenicol (CHL, 30µg), nitrofurantoin (F, 300 ug), amoxicillin/clavulanic acid (AMC, 20/10 µg), tobramycin (TOP,

10ug), meropenem (MER, 10µg), cefotaxime (CTX, 30ug), cefepime (CFP,30 µg), and ceftazidime (CAZ, 30 µg). The antibiotic discs used were the product of Abtek Biologicals Ltd, Liverpool, United Kingdom. Inhibition zones were measured using ruler and isolates were categorized as: resistance, intermediate and susceptible for each antimicrobial agent using the break point as set in line with 29th edition CLSI guidelines (70). The isolates were going to be considered as MDR when they were non- susceptible for three or more classes of antibiotics (71). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853were used for quality control throughout the antimicrobial susceptibility tests as recommended by29th edition CLSI.

5.10. Laboratory Data Quality Assurance

Sample collection, handling, transportation and microbiological analysis and interpretation of results were carried out using standard operating procedures. Before the tangible procedure; reagents, antimicrobial disks, and media were checked for damage, storage problems and expiry date. Laboratory equipment's are appropriately cleaned and sterilized before use. Media's was prepared according to the respective manufacturer's instruction. Five percent of prepared media per batch was incubated overnight for sterility checkup. Quality control for new batch was performed using ATCC 25922 *E. coli* standard control to cross check the quality of antibiotics disks and culture media. For ESBLs confirmatory test *E. coli* ATCC 25922(ESBLs negative) and *K. pneumoniae* ATCC 700603 (ESBLs positive) standard control strains are served at time of the procedure (70).

5.11. Data analysis and interpretation method

Data was entered and summarized using SPSS version 20 software (IBM Corporation, Armonk, NY, USA).Frequency and percentages of isolates, antibiotic susceptibility pattern of Enterobacteriaceae and ESBLs-pE were calculated. Tables and figures have been employed for data demonstration.

5.12. Statistical Data Quality Assurance

Before data entry, data from the data collection form was cross checked for its completeness and accuracy. Culture isolates and antibiotics susceptibility test results had been documented consciously ahead of entry to SPSS. Furthermore, data cleaning and double-data entry was implemented to assure quality of the data.

5.13. Ethical Consideration

- The study proposal was reviewed and approved by the department of research and ethics review committee of the medical laboratory sciences, college of health sciences; Addis Ababa University
- Ethical clearance was obtained from Addis Ababa health bureau research and ethics review committee.
- Permission and ethical clearance was obtained from the respective hospitals in which the samples were collected.

5.14. Dissemination of the result

- The final written report was submitted to
  - ✓ Addis Ababa University, College of Health Science, Department of Medical Laboratory Sciences.
  - ✓ Hospitals participating in the study
  - ✓ Addis Ababa health bureau and with all participated hospitals.
- The finding was presented in seminars by inviting the head of collage of health science and medical laboratory technology (MLT) as well as staff workers of MLT department and any other volunteers to attain the seminar.
- By keeping confidentially, the manuscript was prepared and sent for peer review and will be published at appropriate journals.

5.15. Operational Definitions

Multi-drug resistant (MDR): when a bacteria simultaneously resistance to at least three different classes of antimicrobial agents.

Non susceptible: are those bacteria which are resistant and intermediate for antibiotics susceptibility

Extended spectrum bata-lactamase producing Enterobacteriaceae: are bacteria that produce beta-lactamase enzymes to resist penicillins, first, second, and third generation cephalosporins and monobactams antibiotics by hydrolysis of these antimicrobials, but not cephamycins (e.g. cefoxitin) or carbapenems (meropenem).

Potential (Suspicious) ESBLs producing Enterobacteriaceae: are those bacteria which showed cefotaxime zone of inhibition $\leq$ 27 mm and ceftazidime zone of inhibition $\leq$ 22 mm (viz. ESBLs screening positive)

Non-ESBLs suspicious/potential Enterobacteriaceae: are bacteria that are ESBLs screening negative

Non-ESBLs Enterobacteriaceae: are bacteria including Non-ESBLs suspicious/potential and confirmed ESBLs negative Enterobacteriaceae.

6. Results

6.1. Distribution of Enterobacteriaceae against Sampling Unit and Hospitals

A total of 100 hospital effluent samples were collected and analyzed for the presence of Enterobacteriaceae family. Of these samples 87% were tested positive and contained one or more than one type of isolates. Meanwhile, 183 non-duplicate Gram-negative bacteria were picked from MacConkey agar, 80.3% (147) belonging to Enterobacterial species. The remaining isolates included *Pseudomonas* spp. (2.2%), *Acinetobacter* spp. (4.4%), and other unidentified Gram-negative bacteria (13%).

Of 147 Enterobacteriaceae family isolates recovered, the distributions were from; laboratory unit 32 (21.8%), mixed source 30(20.4%), adult ward 21 (14.3%), labor ward 21 (14.3%), pediatric ward 19 (12.9%) and laundry unit effluent 17 (11.6%) and in addition, 7 (4.8%) isolates were from MDR TB ward effluent, which was only collected from ALERT. In this study, the highest number of isolated bacteria, irrespective of total sample collected, were recovered from Y12HMC (34), MIIRH (32) and SPHMMC (32); while, the least isolates were obtained from TASH (21) (Table 2).

6.2. Frequency of Enterobacteriaceae Isolates

Among all Enterobacteriaceae the most frequent isolates were *E. coli* (45.6 %), *K. pneumoniae* (10.2 %) and *E. cloacae* (9.5%) respectively. *E. coli* was predominantly isolated in laboratory effluent (22.4%) followed by adult ward effluent (19.4%). Meanwhile, *K. pneumoniae* were mostly obtained from laboratory effluent (26.7%), laundry unit (20%) and mixed source wastewater samples (20%).*E. coli* and *K. pneumoniae* were the most frequently isolate identified from pediatric ward (47.4%, 10.5%) and laboratory unit (46.9%, 12.5%) respectively; whereas *E. coli* and C*itrobacter* spp. were predominantly isolated from adult ward, (61.9%, 19.5%) and mixed source effluent (33.3%, 13.3%) respectively (Table 2).

Table 2. Distribution of Enterobacteriaceae isolate against within hospital, sampling unit, time and round of sample collection, at selected governmental hospitals wastewater, Addis Ababa, Ethiopia from April 1 to May 31, 2020.

Variables (n)		Enterobacteriacae isolates n(%)									
		E.coli	***K.pneumonia***	***E.cloacae***	***Citrobacter* spp.**	***K.ozanae***	***K.rhinoscler***	***K.oxytoca***	***M.morganii***	***Sallmonella* spp.**	***Other isolates*** ✱
Hospital	**TASH(21)**	6(28.6)	2(9.5)	4(19)	1(4.8)	3(14.3)	3(14.3)	1(4.8)	0	1(4.8)	0
	SPHMMC(32)	17(53.1)	3(9.4)	2(6.3)	1(3.1)	2(6.3)	1(3.1)	0	1(3.1)	1(3.1)	4(12.5)
	ALERT(28)	12(42.9)	3(10.7)	2(7.1)	4(14.3)	0	0	2(7.1)	2(7.1)	1(3.6)	2(7.1)
	Y12HMC(34)	16(47.1)	3(8.8)	3(8.8)	4(11.8)	1(2.9)	3(8.8)	2(5.9)	1(2.9)	0	1(2.9)
	MIIRH(32)	16(50)	4(12.5)	3(9.4)	2(6.3)	2(6.3)	1(3.1)	2(6.3)	0	0	2(6.3)
Sampling Unit	**Adult ward(21)**	13(61.9)	1(4.8)	2(9.5)	4(19)	0	0	0	0	1(4.8)	0
	Pediatric ward(19)	9(47.4)	2(10.5)	2(10.5)	1(5.3)	2(10.5)	1(5.3)	1(5.3)	1(5.3)	0	0
	Laboratory unit(32)	15(46.9)	4(12.5)	3(9.4)	1(3.1)	1(3.1)	4(12.5)	1(3.1)	1(3.1)	0	2(6.3)
	Laundry unit(17)	10(58.8)	3(17.6)	2(11.8)	0	0	0	0	0	0	2(11.8)
	Labor ward(21)	7(33.3)	2(9.5)	2(9.5)	1(4.8)	1(4.8)	2(9.5)	3(14.3)	0	1(4.8)	2(9.5)
	Mixed source(30)	10(33.3)	3(10)	3(1)	4(13.3)	4(13.3)	1(3.3)	2(6.7)	0	1(3.3)	2(6.7)
	MDR TB ward(7)	3(42.9)	0	0	1(14.3)	0	0	0	2(28.6)	0	1(14.3)
Time of collection	**Morning(71)**	33(46.5)	10(14.1)	7(9.9)	4(5.6)	1(1.4)	4(5.6)	3(4.2)	3(4.2)	3(4.2)	3(4.2)
	Afternoon(76)	34(44.7)	5(6.6)	7(9.2)	8(10.5)	7(9.2)	4(5.3)	4(5.3)	1(1.3)	0	6(7.9)
Round of collection	**First(80)**	36(45)	7(8.8)	8(10)	6(7.5)	5(6.3)	5(6.3)	5(6.3)	2(2.5)	2(2.5)	4(5)
	second(67)	31(46.3)	8(11.9)	6(9)	6(9)	3(4.5)	3(4.5)	2(3)	2(3%)	1(1.5)	5(7.5)
Total (N=147)		**67(45.6)**	**15(10.2)**	**14(9.5)**	**12(8.2)**	**8(5.4)**	**8(5.4)**	**7(4.8)**	**4(2.7)**	**3(2)**	**9(6.1)**

Note; ✱other isolates were *Proteus* spp., *Shigella* spp., *Ent. aerogens, Edwardsella,* and *P. alkalifacience*

TASH=Tikur Ambesa Specialized Hospital, SPHHMMC=St. Paul's Hospital Millennium Medical College, ALERT=All African leprosy and tuberculosis rehabilitation training center, Y12HMC=Yekatit 12 hospital medical college, MIIRH=Menilik II referral hospital, MDR TB Ward = multidrug resistant tuberculosis ward

6.3. Antimicrobial Susceptibility pattern of Enterobacteriaceae

The prevalence of antimicrobial non-susceptible pattern for Enterobacteriaceae isolated ranged from 8.2 to 77.6% in wastewater isolates, with most of the strains susceptible to meropenem (MER) and nitrofruntonine (F).They showed high non-susceptible to amoxicillin-clavulanic acid (77.6%) and trimethoprim/sulfamethoxazole (57.8%). Whereas, meropenem, nitrofurantoin, and tazobactam/piperacillin had the lowest non-susceptible rate 8.2%, 8.2%, 12.9% respectively. In this study the most first, second and third non-susceptible Enterobacteriaceae viz. *E. coli, Citrobacter* spp. and *E. cloacae* revealed the highest non-susceptible for amoxicillin-clavulanic acid of 85.1%, 75%, and 85.7% respectively. However, the fourth most non-susceptible K. *pneumoniae* showed the highest level of non-susceptible against CTX (60%) (Table 3).

Out of 147 Enterobacteriaceae strains tested, 125 (85%) were found non-susceptible to at least one or more antibiotics tested. Hence, 2.7% were non-susceptible to all antibiotics tested (12 drugs), 0.7% non-susceptible to 10 drugs, and 12.9% were non-susceptible to seven drugs (Table 4). In the same talked, 64% (94/147) of tested Enterobacteriaceae were MDR strain. Meanwhile, a total of 62.7% *E. coli*, 83.3%*Citrobacter* spp., and 64.3% *E. cloacae* were identified as the predominant MDR isolates within species (Table 5).

Table 3.Antimicrobial non-susceptible pattern of Enterobacteriaceae identified from different sampling unit at selected governmental hospitals in Addis Ababa, Ethiopia from April 1 to May 31, 2020.

Isolates (number)	**Tested Antibiotics Number n (%))**											
	SXT	**CPR**	**TZP**	**CXT**	**CHL**	**F**	**AMC**	**TOB**	**MER**	**CTX**	**CFP**	**CAZ**
***E.coli*(67)**	43(64.2)	34(50.7)	9(13.4)	14(20.9)	4(6)	0	57(85.1)	18(26.9)	3(4.5)	35(52.2)	32(47.8)	29(43.3)
***K.rhinoscler*(8)**	4(50)	4(50)	0	2(25)	3(37.5)	0	7(87.5)	4(50)	0	4(50)	4(50)	4(50)
***K.pneumonias*pp (15)**	7(46.7)	6(40)	0	0	2(13.3)	0	8(53.3)	5(33.3)	0	9(60)	8(53.3)	8(53.3)
***Citrobacters*pp.(12)**	8(66.7)	7(58.3)	1(8.3)	7(58.3)	5(41.7)	2(16.7)	9(75)	2(16.7)	2(16.7)	6(50)	5(41.7)	6(50)
***Sallmonellas*pp.(3)**	1(33.3)	2(66.7)	0	0	1(33.3)	0	3(100)	1(33.3)	1(33.3)	2(66.7)	2(66.7)	2(66.7)
***E.cloacae*(14)**	7(50)	9(64.3)	5(35.7)	9(64.3)	6(42.9)	6(42.9)	12(85.7)	6(42.9)	5(35.7)	8(57.1)	8(57.1)	8(57.1)
***K.oxytoca*(7)**	2(28.6)	3(42.9)	0	1(14.3)	1(14.3)	0	3(42.9)	2(28.6)	0	3(42.9)	2(28.6)	3(42.9)
***K.ozanae*(8)**	5(62.5)	2(25)	1(12.5)	4(50)	2(25)	0	5(62.5)	3(37.5)	0	5(62.5)	5(62.5)	4(50)
***M.morganii* (4)**	3(75)	3(75)	0	0	0	2(50)	3(75)	0	0	3(75)	3(75)	3(75)
Other isolates *(9)*	5(55.6)	7(77.8)	3(33.3)	2(22.2)	2(22.2)	2(22.2)	7(77.8)	2(22.2)	1(11.1)	4(44.4)	4(44.4)	4(44.4)
Total non-susceptible (147)	**85(57.8)**	**77(52.4)**	**19(12.9)**	**39(26.5)**	**26(17.7)**	**12(8.2)**	**114(77.6)**	**43(29.3)**	**12(8.2)**	**79(53.7)**	**73(49.7)**	**71(48.3)**

Note: SXT= Sulfamethoxazole-trimethoprim, CRP= Ciprofloxacin, TZP= Tazobactam/Piperacillin, CXT= Cefoxitin, CHL= Chloramphenicol, F= nitrofurantoin, AMC= Amoxicillin-Calvulanic acid, TOB= Tobramycine, MER= Meropenem, CTX= Cefotaxime, CFP=Cefepime, CAZ= Ceftazidime. ✱other isolates were *Proteus* spp, *Shigella* spp, *Ent. aerogens*, *Edwardsella*, and *P. alkalifacience*

Table 4.Level of antibiotic non-susceptible of Enterobacteriaceae identified from different sampling units of selected governmental hospitals, Addis Ababa, Ethiopia from April 1 to May 31, 2020.

Isolates (number)	**Level of antibiotics non-susceptible ((number (%))**												
	Ro	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**	**R10**	**R11**	**R12**
E.coli(67)	6(9)	8(11.9)	11(16.4)	8(11.9)	4(6)	7(10.4)	8(11.9)	5(7.5)	4(6)	4(6)	1(1.5)	1(1.5)	0
K.rhinoscler(8)	1(12.5)	0	1(12.5)	2(25)	0	0	1(12.5)	2(25)	1(12.5)	0	0	0	0
K.pneumonia(15)	6(40)	1(6.7)	0	0	0	1(6.7)	2(13.3)	5(33.3)	0	0	0	0	0
Citrobacter spp.(12)	0	0	2(16.7)	2(16.7)	2(16.7)	2(16.7)	1(8.3)	1(8.3)	1(8.3)	0	0	1(8.3)	0
Sallmonella spp.(3)	0	1(33.3)	0	0	0	0	0	2(66.7)	0	0	0	0	0
E.cloacae(14)	2(14.3)	0	3(21.4)	1(7.1)	0	0	1(7.1)	1(7.1)	1(7.1)	0	0	1(7.1)	4(28.6)
K.oxytoca(7)	4(57.1)	0	0	0	0	1(14.3)	0	1(14.3)	1(14.3)	0	0	0	0
K.ozanae(8)	2(25)	0	1(12.5)	0	0	1(12.5)	2(25)	0	1(12.5)	1(12.5)	0	0	0
M.morganii(4)	1(25)	0	0	0	0	0	1(25)	2(50)	0	0	0	0	0
Other isolates (9)	0	0	3(33.3)	1(11.1)	1(11.1)	2(22.2)	0	0	0	1(11.1)	0	1(11.1)	0
TOTAL(N=147)	**22(15)**	**10(6.8)**	**21(14.3)**	**14(9.5)**	**7(4.8)**	**14(9.5)**	**16(10.9)**	**19(12.9)**	**9(6.1)**	**6(4.1)**	**1(0.7)**	**4(2.7)**	**4(2.7)**

Note: R0: non-susceptible to no antibiotics, R1-12: non-susceptible to 1, 2, 3, 4, 5, 6- 12 antibiotics used at time of study..✱other isolates were *Proteus* spp, *Shigella* spp, *Ent. aerogens*, *Edwardsella*, and *P. alkalifacience*

Table 5. Occurrence of MDR and ESBLs producing Enterobacteriaceae spp. Within hospital and sampling units as well as within MDR and ESBLs producer at selected governmental hospitals, in Addis Ababa, Ethiopia from April 1 to May 31, 2020.

Hospitals isolates n, Vs ESBLs positive and MDR yes n(%)			**Sampling unit n(%)**							Wastewater collected time		Wastewater collected round	
			Adult ward	Pediatric ward	Laboratory	Laudnary	Labor ward	Mixed source	MDR TB ward	Morning	Afternoon	First	Second
sample collected hospitals	TASH,21	ESBLs positive 11(52.4)	1(9.1)	2(18.2)	2(18.2)	1(9.1)	2(18.2)	3(27.3)	NC	4(36.4)	7(63.6)	11(100)	NC
		MDR yes 16(31.2)	1(6.3)	4(25)	4(25)	1(6.3)	3(18.8)	3(18.8)	NC	8(50)	8(50)	16(100)	NC
	SPHMMC,32	ESBLs positive 15(46.9)	2(13.3)	3(20)	2(13.3)	4(26.7)	NC	4(26.7)	NC	7(46.7)	8(53.3)	4(26.7)	11(73.3)
		MDR yes 22(68.8)	4(18.2)	3(13.6)	4(18.2)	5(22.7)	NC	6(27.3)	NC	11(50)	11(50)	9(40.9)	13(59.1)
	ALERT,28	ESBLs positive 19(67.9)	4(21.1)	NC	6(31.6)	1(5.3)	4(21.1)	NC	4(21.1)	9(47.7)	10(52.6)	12(63.2)	7(36.8)
		MDR yes 20(71.4)	3(15)	NC	6(30)	1(5)	4(20)	NC	6(30)	10(50)	10(50)	11(55)	9(45)
	Y12HMC,34	ESBLs positive 16(47.1)	4(25)	2(12.5)	1(6.3)	0	3(18.8)	6(37.5)	NC	8(50)	8(50)	9(56.3)	7(43.8)
		MDR yes 19(55.9)	4(21.1)	3(15.8)	1(5.3)	0	4(21.1)	7(36.8)	NC	8(42.1)	11(57.9)	9(47.4)	10(52.6)
	MIIRH,32	ESBLs positive 10(31.2)	3(30)	0	2(20)	4(40)	1(10)	0	NC	6(60)	4(40)	3(30)	7(70)
		MDR yes 17(53.1)	3(17.6)	0	5(29.4)	5(29.4)	2(11.8)	2(11.8)	NC	8(47.1)	9(52.9)	7(41.2)	10(58.8)
Total, N=147		**ESBLs positive N=71(48.3%)**	**14(19.7)**	**7(9.9)**	**13(18.3)**	**10(14.1)**	**10(14.1)**	**13(18.3)**	**4(5.6)**	**34(47.9)**	**37(52.1)**	**39(54.9)**	**32(45.1)**
		MDR yes N=94(64%)	**15(16)**	**10(10.6)**	**20(21.3)**	**12(12.8)**	**13(13.8)**	**18(19.1)**	**6(6.4)**	**45(47.9)**	**49(52.1)**	**52(53.3)**	**42(44.7)**

Note: TASH=Tikur Ambesa Specialized Hospital, SPHHMMC= St. Paul's Hospital Millennium Medical College, ALERT= All African leprosy and tuberculosis rehabilitation training center, Y12HMC= Yekatit 12 hospital medical college, MIIRH= Menilik II referral hospital, MDR TB Ward = multidrug resistant tuberculosis ward, NC= sample not collected

6.4. Magnitude of ESBLs Producing Enterobacteriaceae

Of all Enterobacteriaceae 55.1% (81/147) were suspected as potential ESBLs producing with screening method of cefotaxime zone of inhibition ≤ 27 mm and ceftazidime zone of inhibition ≤ 22 mm (

Figure **1**). Out of 81 ESBLs potential Enterobacteriaceae, 71 (87.7%) were found to be ESBLs producing isolates by the combination disk test. The overall magnitude of ESBLs producing Enterobacteriaceae (ESBLs-pE) were 48.3 % (71/147,) with the highest percentage found in *E. coli*, (21.8%), *K. pneumoniae* (4.8%) and *Citobacter* spp. (4.8%); while, the lowest ratio observed in *Salmonella* spp. 1.4% (2/147) respectively (Figure 2). The occurrence of ESBLs producers differ strongly within different species of Enterobacteriaceae. The highest within-species frequency of ESBLs production was recovered among *M. morganii* (75%) pursued by *Salmonella* spp (66.7%) and *K .ozanae* (62.5%) respectively. However, least within-species ESBLs production was found in *E. cloaca* (21%) (Table 6 and Figure 2).

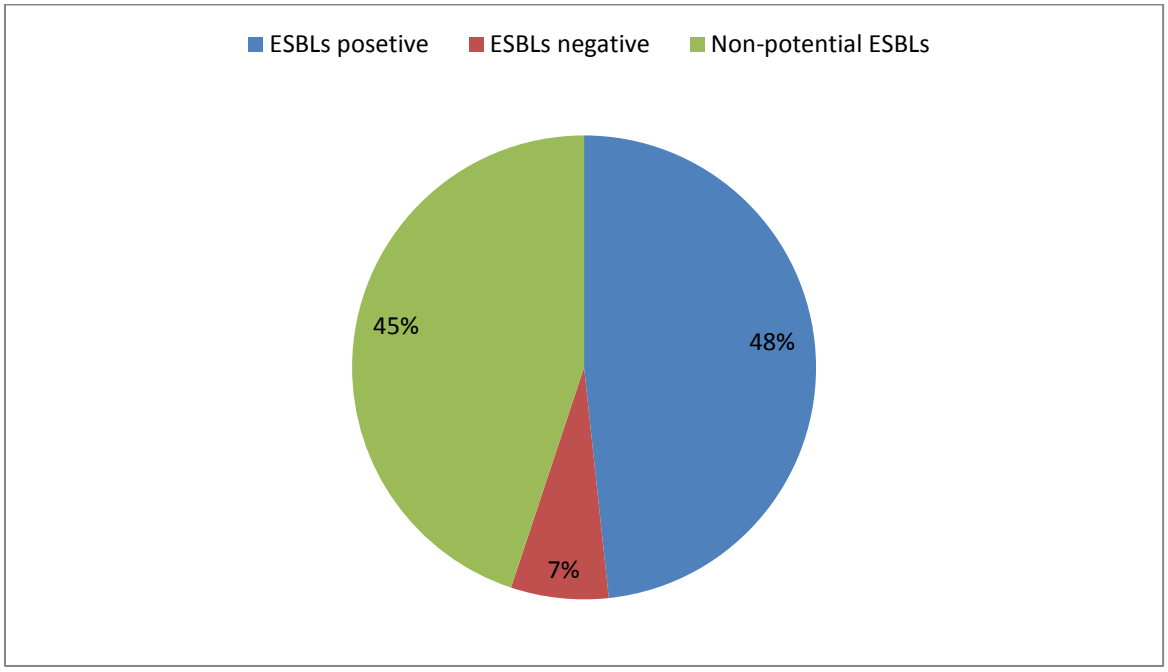

Figure 1.The percentage of ESBLs positive, ESBLs negative and non-potential ESBLs Enterobacteriaceae at selected governmental hospitals wastewater, Addis Ababa, Ethiopia April 1 to May 31, 2020.

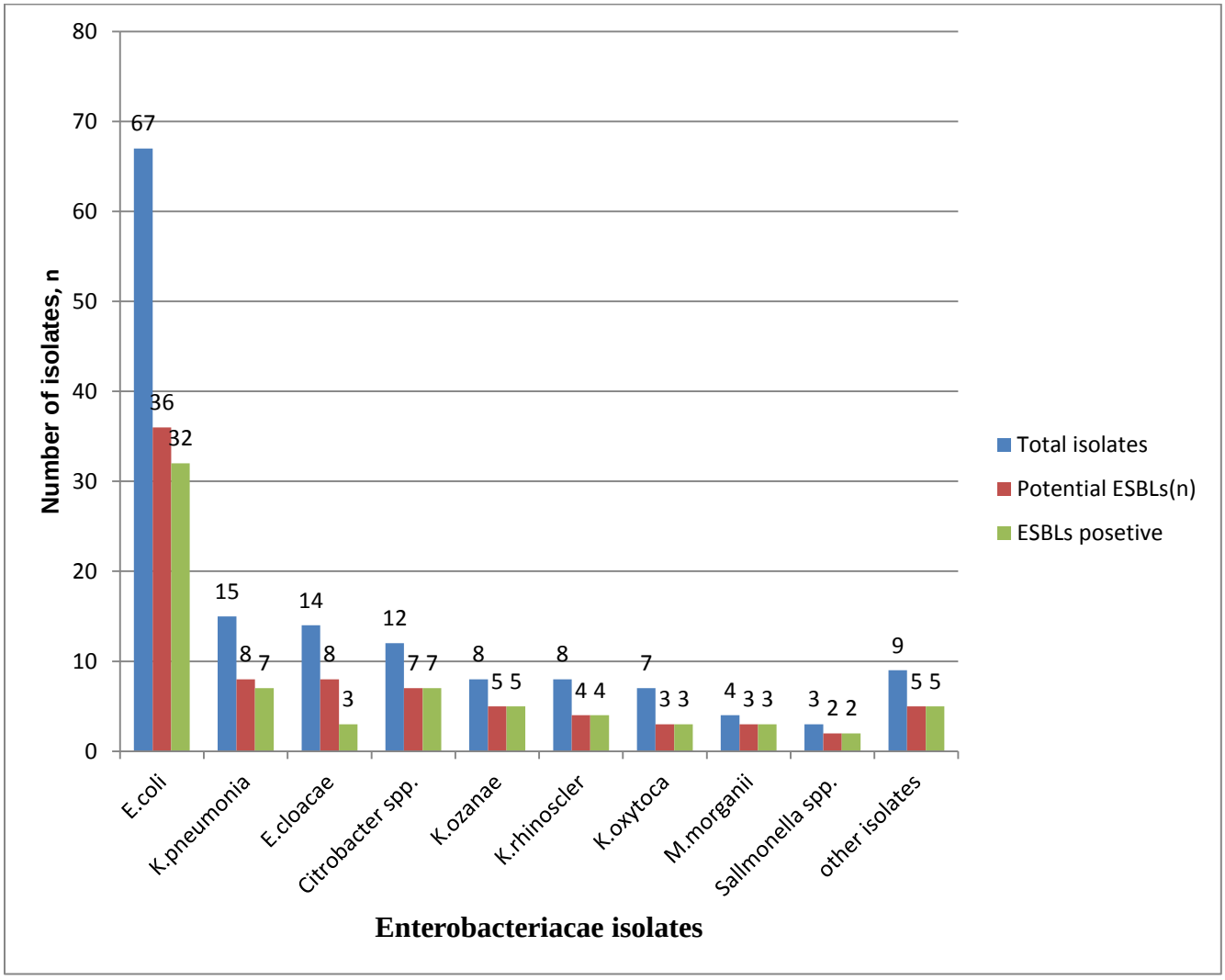

Figure 2.Frequency of total, potential and confirmed ESBLs producing Enterobacteriaceae species from different sampling units at selected governmental hospitals wastewater, in Addis Ababa, Ethiopia from April 1 to May 31, 2020.

Table 6. Prevalence of ESBLs producing Enterobacteriaceae species within hospital, sampling unit, time and round of wastewater collection as well as with in species and ESBLs producer at selected governmental hospitals, in Addis Ababa, Ethiopia from April 1 to May 31, 2020.

ESBL POSETIVE, n(%)		***Enterobacteriaceae* isolate identified n(%)**									
		E.coli	*K.pneumonia*	*Citrobacter* spp.	*K.ozanae*	*K.rhinoscler*	*E.cloacae*	*K.oxytoca*	*M.morgani*	*Sallmonella* spp	other isolates
wastewater collected hospitals	**TASH, 11(52.4)**	2(18.2)	2(18.2)	1(9.1)	2(18.2)	2(18.2)	0	1(9.1)	0	1(9.1)	0
	SPHMMC, 15(46.9)	7(46.7)	1(6.7)	0	2(13.3)	0	0	0	1(6.7)	1(6.7)	3(20)
	ALERT, 19(67.9)	9(47.7)	2(10.5)	2(10.5)	0	0	1(5.3)	2(10.5)	2(10.5)	0	1(5.3)
	Y12HMC, 16(47.1)	7(43.8)	1(6.3)	4(25)	1(6.3)	1(6.3)	1(6.3)	0	0	0	1(6.3)
	MIIRH, 10(31.3)	7(70)	1(10)	0	0	1(10)	1(10)	0	0	0	0
wastewater sampling unit	**Adult ward, 14(66.7)**	9(64.3)	0	3(21.4)	0	0	2(14.3)	0	0	0	0
	Pediatric ward, 7(36.8)	3(42.9)	1(14.3)	1(14.3)	2(28.6)	0	0	0	0	0	0
	Laboratory, 13(40.6)	5(38.5)	2(15.4)	1(7.7)	1(7.7)	1(7.7)	0	1(7.7)	1(7.7)	0	1(7.7)
	Laundry, 10(58.8)	7(70)	2(20)	0	0	0	0	0	0	0	1(10)
	Labor ward, 10(47.6)	2(20)	0	1(10)	1(10)	2(20)	1(10)	1(10)	0	1(10)	1(10)
	Mixed source,13(43.3)	4(30.8)	2(15.4)	1(7.7)	1(7.7)	1(7.7)	0	1(7.7)	0	1(7.7)	2(15.4)
	MDR TB ward, 4(57.1)	2(50)	0	0	0	0	0	0	2(50)	0	0
wastewater collected time	**Mornining, 34(47.9)**	15(44.1)	5(14.7)	2(5.9)	1(2.9)	2(5.9)	1(2.9)	1(2.9)	2(5.9)	2(5.9)	3(8.8)
	Afternoon, 37(48.7)	17(45.9)	2(5.4)	5(13.5)	4(10.8)	2(5.4)	2(5.4)	2(5.4)	1(2.7)	0	2(5.4)
wastewater collected round	**First, 39(48.8)**	17(43.6)	3(7.7)	3(7.7)	3(7.7)	3(7.7)	1(2.6)	3(7.7)	2(5.1)	2(5.1)	2(5.1)
	Second, 32(47.8)	15(46.9)	4(12.5)	4(12.5)	2(6.3)	1(3.1)	2(6.3)	0	1(3.1)	0	3(9.4)
TOTAL	**Total ESBL Pos N=71**	**32(45.1)**	**7(9.9)**	**7(9.9)**	**5(7)**	**4(5.6)**	**3(4.2)**	**3(4.2)**	**3(4.2)**	**2(2.8)**	**5(7)**
	Total Isolates N=147	**67(47.8)**	**15(46.7)**	**12(58.3)**	**8(62.5)**	**8(50)**	**14(21.4)**	**7(42.9)**	**4(75)**	**3(66.7)**	**9(71.4)**

Note: TASH=Tikur Ambesa Specialized Hospital, SPHHMMC= St. Paul's Hospital Millennium Medical College, ALERT= All African leprosy and tuberculosis rehabilitation training center, Y12HMC= Yekatit 12 hospital medical college, MIIRH= Menilik II referral hospital, MDR TB Ward = multidrug resistant tuberculosis ward

6.5. Distribution of MDR and ESBLs producing Enterobacteriaceae against the variables

From all hospital wastewater collected for this study purpose, the highest ratio of ESBLs-pE within sampling unit was observed in adult ward effluent (66.7%) followed by laundry unit (58.8%) and labor ward (47.6%) respectively; whereas, the least proportion was recovered in pediatric ward (36.9%) and laboratory unit effluent (40.6%) (Table 6). Similarly with less difference; the elevated MDR isolates within sampling unit were identified in adult ward effluent (71.4%) pursued by laundry unit (70.6%) and laboratory unit (62.5%) correspondingly; while the lowest ratio was found, in pediatric ward (52.6%) (Table 7). In MDR TB ward wastewater which was collected only in ALERT hospital, the proportions of ESBLs-producing and MDR Enterobacteriaceae within sampling unit were 57.1% and 85.7% respectively (Table 6 and Table 7). Generally, of all MDR Enterobacteriaceae, 73.4% (69/94) were ESBLs producer, whereas only 26.6% (25/94) of them were non-ESBLs producer Enterobacteriaceae.

The magnitude of ESBLs producing and MDR Enterobacteriaceae in the wastewater were different in the five hospitals. The highest occurrence of ESBLs-pE within hospital according to CDT identification method was found in wastewater from the ALERT (67.9%), followed by TASH (52.4%) and Y12HMC (47.1%) respectively. Whereas the least ESBLs-pE within hospital occurred in MIIRH (31.2%) (Table 6). In contrary, the elevated MDR isolates within hospital were observed in wastewater of TASH (76.2%) and ALERT (71.4%); while, the lowest ratio was found in the same way as ESBL producer isolates in MIIRH (53.1%) (Table 7).

The magnitude of MDR and ESBLs-pE obtained from all wastewater samples were higher at the afternoon than the morning wastewater collected. At morning effluent the occurrence of MDR isolates and ESBL producers within time of wastewater collection were 59.2% and 47.9%, whereas at the afternoon were 64.5% and 52.1% respectively. On the contrary, they were higher in the first round of effluent collection than the second. In the first round MDR isolates and ESBL producers were 77.6% and 48.8%, while in the second round effluent collection they were 62.7% and 40% correspondingly (Table 6 and Table 7).

Table 7. Prevalence of MDR Enterobacteriaceae species within hospital, sampling unit, time and round of wastewater collection as well as within species and MDR isolates at selected governmental hospitals, in Addis Ababa, Ethiopia from April 1 to May 31, 2020.

MDR YES, n (%)		Enterobacteriacae isolates recoverd from hospital effluents n(%)									
		E. coli	*Citrobacter* spp.	*E. cloacae*	*K. pneumonia*	*K. rhinoscler*	*K. ozanae*	*K. oxytoca*	*M. morganii*	*Sallmonella*s pp.	Other isolates
wastewater collected Hospitals	**TASH, 16(76.2)**	3(18.8)	1(6.3)	4(25)	2(12.5)	2(12.5)	2(12.5)	1(6.3)	0	1(6.3)	0
	SPHMMC, 22(68.8)	10(45.5)	1(4.5)	1(4.5)	2(9.1)	1(4.5)	2(9.1)	0	1(4.5)	1(4.5)	3(13.6)
	ALERT, 20(71.4)	9(45)	3(15)	1(5)	2(10)	0	0	2(10)	2(10)	0	1(5)
	Y12HMC, 19(55.9)	9(47.4)	4(21.1)	1(5.3)	1(5.3)	2(10.5)	1(5.3)	0	0	0	1(5.3)
	MIIRH, 17(53.1)	11(64.7)	1(5.9)	2(11.8)	1(5.9)	1(5.9)	0	0	0	0	1(5.9)
wastewater sampling unit	**Adult ward, 15(71.4)**	9(60)	3(20)	2(13.3)	1(6.7)	0	0	0	0	0	0
	Pediatric ward,10(52.6)	5(50)	1(10)	1(10)	1(10)	0	2(20)	0	0	0	0
	Laboratory, 20(62.5)	7(35)	1(5)	2(10)	2(10)	3(15)	1(5)	1(5)	1(5)	0	2(10)
	Laundry, 12(70.6)	8(66.7)	0	1(8.3)	2(16.7)	0	0	0	0	0	1(8.3)
	Labor ward, 13(61.9)	4(30.8)	1(7.7)	2(15.4)	0	2(15.4)	1(7.7)	1(7.7)	0	1(7.7)	1(7.7)
wastewater collected Time	**Morning, 45(63.4)**	18(40)	3(6.7)	5(11.1)	6(13.3)	4(8.9)	1(2.2)	1(2.2)	2(4.4)	2(4.4)	3(6.7)
	Afternoon, 49(64.5)	24(49)	7(14.3)	4(8.2)	2(4.1)	2(4.1)	4(8.2)	2(4.1)	1(2)	0	3(6.1)
wastewater collected round	**First, 52(65)**	22(42.3)	5(9.6)	5(9.6)	4(7.7)	3(5.8)	3(5.8)	3(5.8)	2(3.8)	2(3.8)	3(5.8)
	Second, 42(62.7)	20(47.6)	5(11.9)	4(9.5)	4(9.5)	3(7.1)	2(4.8)	0	1(2.4)	0	3(7.1)
TOTAL	**Total MDR N=94**	**42(44.7)**	**10(10.6)**	**9(9.6)**	**8(8.5)**	**5(6.4)**	**5(5.3)**	**3(3.2)**	**3(3.2)**	**2(2.5)**	**6(6.4)**
	Total Isolate N=147	**67(62.7)**	**12(83.3)**	**14(64.3)**	**15(53.3)**	**8(62.5)**	**8(62.5)**	**7(42.9)**	**4(75)**	**3(66.7)**	**9(66.7)**

Note: TASH=Tikur Ambesa Specialized Hospital, SPHHMMC= St. Paul's Hospital Millennium Medical College, ALERT= All African leprosy and tuberculosis rehabilitation training center, Y12HMC= Yekatit 12 hospital medical college, MIIRH= Menilik II referral hospital, MDR TB Ward = multidrug resistant tuberculosis ward

6.6. Antibiotics Susceptibility pattern of ESBLs producing Enterobacteriaceae to potentially active drugs

The antibiotic non-susceptible profile for ESBLs producing and non ESBLs producer Enterobacteriaceae are displayed in Figure 3 below. The antibiotics susceptibility pattern of ESBLs-pE were also performed in relation to potential active antibiotics like carbapenems (meropenem), quinolone/fluroquinolone (ciprofloxacine), cephamycine (cefoxitin) and aminoglycoside (tobramycin) drug Categories. In addition, it was tested against for combinantion drug (amoxaciline-clavunalate and pepracillin-tazobactam), phenicol (chloramphenicol), nitrofuran (nitrofurantoin) and folate pathway antagonist (trimthoprime-sulfamethoxazole) drug families.

The predominant ESBLs-pE was found to be more than 85% non-susceptible, the highest non-susceptible levels were observed on cefotaxime (95.8%), amoxacilline/clavunalate (93%) and cefipeme (90.1%). The most common co-resistance rates among the ESBLs-pEisolates to ciprofloxacin and trimthoprime-sulfamethoxazole were 74.6% and 73.2% respectively. Meanwhile, aminoglycoside and cephamycine such as tobramycine (40.8%) and cefoxitine (29.6%) showed reduced efficacy against the ESBLs-pE. However, the most active drugs for ESBLs producing isolates were meropenem, nitrofurantoin and piperacillin/tazobactam with susceptibility 94.4%, 88.7% and 87.3% correspondingly. Out of confirmed ESBLs-pE the highest ESBLs production was observed among *E.coli* with 45% (32/71) prevalence. While, the least ESBLs production from the total ESBLs positive Enterobacteriaceae was detected in *salmonella* spp. with 2.2% (2/71) ratio (Table 6).

Non-ESBLs producers Enterobacteriaceae showed non-susceptible level of 63.2%, 43.4% and 31.6% to amoxacilline/clavunalate, trimthoprime-sulfamethoxazole and ciprofloxacin respectively. In addition, nitrofurantoin, chloramphenicol and meropenem showed least non-susceptible with 7.9%, 10.5 and 10.5% respectively.

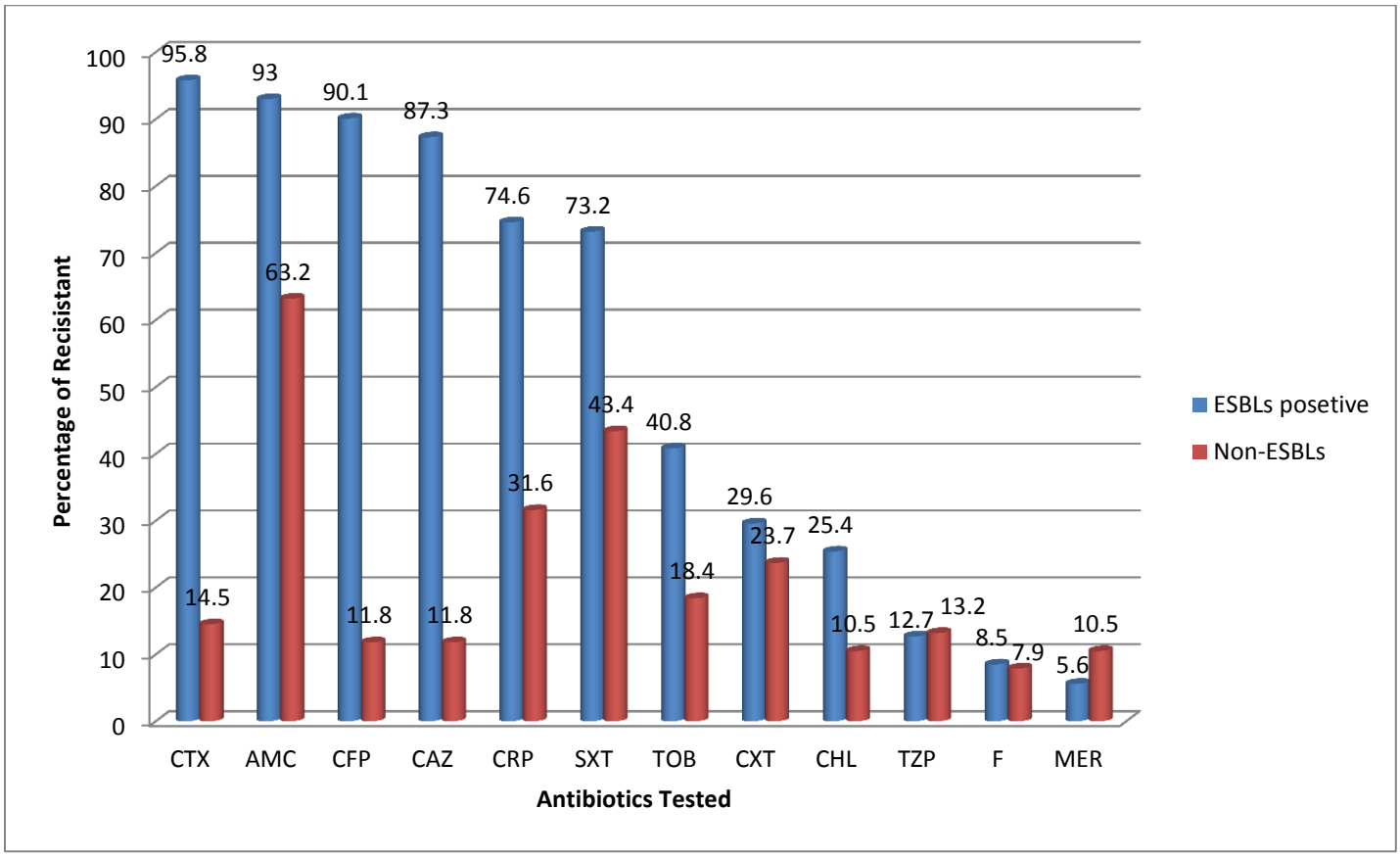

Figure 3. Antibiotics Non-susceptible pattern of ESBLs positive and Non-ESBLs Enterobacteriaceae to different classes of antibiotics at selected governmental hospitals, Addis Ababa, Ethiopia April 1 to May 31, 2020.

Note: SXT= Sulfamethoxazole-trimethoprim, CRP= Ciprofloxacin, TZP= Tazobactam/Piperacillin, CXT= Cefoxitin, CHL= Chloramphenicol, F= nitrofurantoin, AMC= Amoxicillin-Calvulanic acid, TOB= Tobramycine, MER= Meropenem, CTX= Cefotaxime, CFP=Cefepime, CAZ= Ceftazidime.

7. Discussion

The occurrence of antibiotic resistant and ESBLs-pE from hospital wastewater could be exceptionally problematic because of the ability of nosocomial pathogens to transfer antibiotic resistance genes among different hosts and environments (72). The dissemination of MDR bacteria via hospital wastewater is a usable cause for concern (73), because it is reasonable that MDR bacteria are selected mostly in hospitals and take away by wastewater. This study, explains about the prevalence, the occurrence of MDR and ESBLs-pE from the five selected governmental hospitals wastewater in Addis Ababa, Ethiopia.

7.1. Prevalence of Enterobacteriaceae isolates

In the present study the most frequent Enterobacteriaceae isolates were *E. coli* (45.6 %), *K. pneumoniae* (10.2 %) and *E. cloacae* (9.5%). Our findings were comparable figure to other studies, in Addis Ababa; *E. coli* (32%), *K. pneumonia* (15%) and *E. cloacae* (6%) (63), in South Eastern, Nigeria; *E.coli* (26.2%) (58), in Luzhou City in Sichuan province, China; *E. coli* (56.5%), *K. pneumoniae* (27.4%) and *Enterobacter* spp.(8.1%) (55), in Bangladish*; E. coli* and *Klebsiella* spp.(30.7% each) and *Enterobacter* spp. (25%) (53).

However, our finding was a little dissimilar to other studies conducted, in Northwest Ethiopia; from hospital environment *Klebsiella* spp. (29.2%), *E. coli* (12.3%)and *Enterobacter* spp. (3.1%) (60), in Mekelle: from untreated hospital wastewater *Klebsiella* spp. (25.9%), and *E.coli* (21.2%) (62), in Biratnagar, Nepal; from effluents of different hospitals sewage *E .coli* (34.7%), *Citrobacter* (21.7%), *Enterobacte*r (21.7%), and *Klebsiella* (13%) (54). These variations might be due to sample type (inanimate object and swage of hospital), study period, sample size and type of pathogen infecting patients at time of sample collection.

7.2. Antibiotics Non-susceptible pattern of Enterobacteriaceae isolates

In the present study, the overall prevalence of antimicrobial non-susceptible pattern for Enterobacteriaceae isolated ranged from 8.2 to 77.6% in wastewater isolates, with most of the strains susceptible to meropenem (MER) and nitrofruntonine (F). This finding was in line with study conducted in Rio de Janeiro, Brazil, with 0 to 83% resistant range for Gram negative isolates and most strains susceptible to meropenem(47).

In this study, out of 147 Enterobacteriaceae strains tested, 125 (85%) were found non-susceptible to at least one or more antibiotics tested. Meanwhile, Enterobacteriaceae strains showed the highest non-susceptible to amoxicillin-clavulanic acid (77.6%) followed by trimethoprim/sulfamethoxazole (57.8%), cefotaxime (53.7%), and ciprofloxacin (52.4%).

This finding more or less correlates with other study conducted in China; trimethoprim/sulfamethoxazole (77.4%), amoxicillin-clavulanic acid (66.1%), and ciprofloxacin (61.3%) (55) had relatively higher resistance. However, our finding disagrees with previous study conducted where lower non-susceptible proportion reported, in Northwest Ethiopia (Gondar): trimethoprim/sulfamethoxazole (29.8%), cefotaxime (23.8%), ciprofloxacin (10.6%) (60), and in Bangiladish; ciprofloxcin (23%) (53).

A study done in China, showed high resistance of Enterobacteriaceae for cefotaxime (100%), meropenem (51.6%) and chloramphenicol (48.4%) (55), contradicting the results presented herein where less non-susceptible observed for cefotaxime (53.7%),chloramphenicol (17.7%) and meropenem (8.2%), again in Rio de Janeiro, Brazil: from the influent wastewater Gram negative isolates showed resistant against cefotaxime (44%), trimethoprim/sulfamethoxazole (34%), ciprofloxacine (17%), and meropenem (3%) (47), which were slightly deviate from current study except meropenem.

In present study, MDR strains were mostly observed in the tested Enterobacteriaceae isolates by 64%.Almost similar MDR isolate results with ours were recorded in study carried out, in Northwest Ethiopia (Gondar) 81.5% (from hospital environment) (60), in Mekelle: 61.5% (from untreated hospital wastewater) (62), in Biratnagar, Nepal; 69.6% (54), in China: 85.5% (55). However, our report contradicted by the previous study conducted in South Eastern, Nigeria (from three hospital effluents) where all the Enterobacteriaceae isolates recovered (*E. coli* and *Salmonella* spp) were MDR although their patterns of resistance varied (58). In the same talked in this study, a total of 83.3% *Citrobacter* spp., 64.3 % *E. cloacae*,

62.7% *E. coli*, and 53.3% *K. pneumoniae* isolates were identified as the predominant MDR. Our finding, was concordant with other previous studies where the common MDR isolates were, in Addis Ababa; *Citrobacter* (100%), *E. cloacae* (66.7%) and *E. coli* (28.6%) (63), in Biratnagar, Nepal; *Enterobacter* spp. (100%), *Citrobacter* spp. (80%), *E. coli* (62.5%), *Klebsiella* spp (33.3%) (54). However, our finding dissimilar with a study carried out in China (*E. coli* (91.4%) and *K. pneumoniae* (94.1%)) (55), Ibadan, Nigeria (*E. coli* (94.8%)) (74), and in Biratnagar, Nepal (*Enterobacter* spp. (100%)) (54), where the highest MDR proportion for *E. coli, K. pneumonia* and *Enterobacter* spp. were indicated.

7.3. Magnitude of Extended Spectrum B-lactamase producing Enterobacteriaceae

In the present study, of all Enterobacteriaceae 55.1% were suspected as potential ESBLs producing and 87.7% of them were confirmed ESBLs producing isolates. A little comparable result was reported in Dubai, UAE by Khan MA. *et al* 2020: among all isolates from municipality wastewater 57.4% suspicious and 25.7% confirmed ESBLs producer Enterobacteriaceae were reported (56), in Northern Italy: 45.4% beta-lactamases producing Enterobacteriaceae were recovered from WWTPs (49). The difference with our result might be due to the type of sample used (hospital Vs municipality wastewater), method used to confirm potential ESBLs producer (CDT Vs DDST) and sample size.

According to the present study, the overall magnitude of ESBLs-pE were 48.3 % which is almost in line with a study conducted in Rio de Janeiro, Brazil (47), and Nepal (54) with ESBLs producer isolates of 39%, and 30.4% respectively. In contrast to the current study, other studies conducted in Ethiopia and other countries reported lower prevalence of ESBLs-pE, in Addis Ababa: 25% from hospital wastewater (63), in Northwest , Ethiopia: 14.8% from hospital environment (61), and in Austria: 27.4% from activated sludge (75), were recovered (75). The difference in the prevalence of ESBLs producer in different studies from wastewater isolates might be due to difference in geographic areas, source of sample, period of study (ESBL rapidly changing over time), sample size, method of ESBL detection and an infection control system.

In the current study, the highest percentage of ESBLs-pE was detected in *E. coli* (45.1%), *K. pneumoniae* (9.9%), and *Citobacter* spp. (9.9%) that is comparable results to study conducted in, Austria with *E. coli* (65.6%), and *K. pneumoniae* (22.6%) (75). and in Ibadan, Nigeria *E.coli*

(29.3%) (74). However, the highest ESBLs producer prevalence was documented in *K. pneumoniae* than *E. coli* or other Enterobacteriaceae spp. in other studies which contradict with the present study. Hence, the predominate ESBLs-pE isolate in other studies were, in Addis Ababa; *Citrobacter* spp. (33.3%), *K.pneumonia* (33.3%), and *E.coli* (20%) (63), in Northwest, Ethiopia, *K. pneumoniae* (42.10%), and *E. coli* (35.09%) (61), in Rio de Janeiro, Brazil: *K. pneumonia* (41.5%), and *E. coli* (12.2%) (47), and in Nepal; *Enterobacter* spp. (60%), *Citrobacter* spp. (40%) and *E. coli* (25%) (54). This variation is occurred because of the difference of, wastewater type (hospital Vs municipality), source of wastewater contaminant, the prevalence of microbes, geographical location and disease epidemiology.

The occurrence of ESBLs producers differ strongly within different species of Enterobacteriaceae. The highest within-species frequency of ESBLs production was recovered among *M. morganii* (75%) pursued by *Salmonella* spp. (66.7%) and *K. ozanae* (62.5%) respectively. Meanwhile, least within-species ESBLs production was found in *E. cloaca* (21%). The difference of the occurrence of ESBLs producer between within ESBLs producer and within species might be the variation of, the number of isolate recovered from the sample, and the isolate compared with (that is comparison within among ESBLs producer Enterobacteriaceae Vs within among each species).

7.4. Distribution of MDR AND ESBLS Producing Enterobacteriaceae against the Independent Variables

In the present study, of all MDR Enterobacteriaceae, 73.4% were ESBLs producer, whereas only 26.6% of them were non-ESBLs producer Enterobacteriaceae. The magnitude of ESBLs producing and MDR Enterobacteriaceae in the wastewater were different in the five hospitals. The highest occurrence of ESBLs-pE within hospital according to CDT identification method were found in wastewater from the ALERT (67.9%), followed by TASH (52.4%) and Y12HMC (47.1%) respectively, whereas; the least ESBLs-pE were detected in MIIRH (31.2%). In contrary; the elevated MDR isolates within hospital were observed in wastewater of TASH (76.2%) and ALERT (71.4%), while; the lowest ratio was found in the same way as ESBL producer isolates in MIIRH (53.1%). It is difficult to compare directly the occurrence of MDR and ESBLs-pE in hospital effluent from one country to other because of the presence of difference in geographical zone, the epidemiology of disease (the severity and disease type), the

number of patients served, the service provided in the hospital and wastewater disposal police from country to country. However, like our country there were different ESBLs producer occurrence within country hospital effluent, in Ibadan, Nigeria: more ESBLs producer was found in a privately-owned hospital (33.3%) than a State Government-owned hospital (29.1%) (74), in Europe: the elevated ESBLs-pE was found in effluents from the Slovenian general hospital, followed by the Austrian private rehabilitation clinic and the Austrian private surgery clinic (51).

From all hospital wastewater collected for this study purpose, the highest ratio of ESBLs-pE within sampling unit was observed in adult ward effluent (66.7%) followed by laundry unit (58.8%) and labor ward effluents (47.6%) respectively; whereas, the least proportion was recovered in pediatric ward (36.9%) and laboratory unit effluent (40.6%). Similarly with less difference; the elevated MDR isolates within sampling unit were identified in adult ward effluent (71.4%) pursued by laundry unit (70.6%) and laboratory unit effluents (62.5%) correspondingly, while; the lowest ratio was found in pediatric ward effluent (52.6%). In MDR TB ward wastewater which was collected only in ALERT hospital, the proportions of ESBLs-producing and MDR Enterobacteriaceae within sampling unit were 57.1% and 85.7% respectively. Almost all the preceding publication on antibiotic resistant profile of pathogenic microbes has been focused towards crude hospital wastewater rather than at each refined source of it. As a result, it was difficult to compare our result directly with other studies conducted from hospital wastewater, anyway a study conducted in hospital environment in Gondar, reported from inanimate object of medical ward, surgical ward and Gyn-obs ward ESBLs-pE of 52.6%, 10.5% and 5.3% respectively (61). The variation in ESBLs-producing and MDR Enterobacteriaceae proportion within sampling unit in the present study might probably be attributed to the difference in type of patients served, length of patient stay, type of medical service provided, infection prevention and control procedure in each department/unit.

In this study, the magnitude of MDR and ESBLs-pE obtained from all wastewater samples were higher at the afternoon than the morning wastewater collected. At the afternoon effluent, the occurrence of MDR and ESBL producer isolates within time of wastewater collection were 64.5% and 52.1%, whereas; at the morning effluent, were 59.2% and 47.9% respectively. This difference most probable happen because of the majority medical activity performed around the afternoon and outpatients number increase at the afternoon due to

transportation and other reasons. On the other hand, MDR and ESBLs-pE were higher in the first round of effluent collection than the second. In the first round, they were 77.6% and 48.8%, while in the second round effluent collection, they were 62.7% and 40% correspondingly.

7.5. Antibiotics susceptibility pattern of ESBLs producing Enterobacteriaceae

In current study, the predominant ESBLs-pE were found to be more than 85% non-susceptible to the antibiotic like cefotaxime (95.8%), AMC (93%), cefipeme (90.1%), and ceftazidime (87.3%). These were in close agreement with other study done in, Northwest Ethiopia; amoxicillin/clavulanic acid (100%) and ceftazidime (100%), Dubai, UAE; from municipality wastewater, cefotaxime (86%) and ceftazidime (77%) (56), Austria; amoxicillin/clavulanic acid (53.1%) (75) had higher resistance for ESBLs-pE.

In this study, the most common higher co-resistant rates among the ESBLs-pE isolates were 74.6% for ciprofloxacine and 73.2% for SXT. Whereas, aminoglycoside and cephamycine such as tobramycine (40.8%) and cefoxitine (29.6%) showed reduced efficacy against the ESBLs-pE. Our result was comparable with other studies taken place, in Northwest Ethiopia; ciprofloxacin (43.9%), and trimethoprim/sulfamethoxazole (SXT) (64.9%) (61), in Austria; ciprofloxacin (56.3%), trimethoprim/sulfamethoxazole (50%) and cefoxitin (25%) (75).

In our work, nearly the most active drugs for ESBLs producing isolates observed are meropenem, nitrofurantoin, and piperacillin/tazobactam with susceptibility 94.4%, 88.7%, and 87.3% and 74.6% respectively. The findings of this study nearly concordance with prior reports conducted in Austria; meropenem (100%), and piperacillin/tazobactam (90.6%) had good susceptibility level (75).

Non-ESBLs producers Enterobacteriaceae were 63.2%, 43.4%, 31.6% and 23.7% non-susceptible to amoxicillin/clavulanic acid, trimethoprim/sulfamethoxazole, ciprofloxacin and Cefoxitin respectively. However, nitrofurantoin, chloramphenicol and meropenem showed least non-susceptible with 7.9%, 10.5 and 10.5% respectively. We observed in the present study ESBLs producer isolates were more resistant to the tested antibiotics than non-ESBLs producer Enterobacteriaceae. This difference might be from the resistant gene on ESBLs producer also contributed the isolate to develop resistance to other antibiotic too.

Strength of the Study

Our study tried to collect the wastewater, at their utmost source, which enables to take preventive measure and at large sample size (in relative to previous study) to be representative. This study conducted at different governmental higher hospitals to display the extent of distribution of MDR and ESBLs-pE in each hospital sampling units/sites.

Limitation of the Study

In this study only wastewater was used, hence it was unable to differentiate the source of non-susceptible bacteria either it was from clinical isolates or sewage system.

Our study was unable to select all antimicrobial agents commonly used for resistance evaluation due to the fact that some of them were not available during the period of study.

The major limitation of the study was that ESBL detection was only performed phenotypically using CDT method, it was better to include genotypic method of detection.

Some source of hospital wastewaters were not incorporated in the study. So, to generalize the distribution of MDR and ESBLs-pE in hospital wastewater, it was better assessing all source of wastewater in selected hospitals.

It would have a better figurative data if the study was also include private hospitals wastewater found in Addis Ababa and WWTP of the city.

8. Conclussion

The hospital wastewater released directly into urban sewerage systems without appropriate disinfection or treatment is the serious environmental attention of the day. In this study, there was high magnitude of MDR & ESBLs-pE (≈65% Vs 50 %) from the wastewater of selected governmental hospitals in Addis Ababa, Ethiopia. Majority of them were in adult ward and laundry unit effluents. In addition, the most frequent ESBLs-pE were *E .coli*, *K. pneumoniae* and *citrobacter* spp. In addition, ESBLs-pE showed high rate of resistance to all tested antibiotics as compared to non-ESBLs-E. This is a warning threat to such infection via contamination of food and water from rivers and urbane drainage which will be polluted through untreated hospital wastewater. Hence, infection prevention and control implementation at each hospital is mandatory

One of the persistence concerns due to the existence of ESBL-producing bacteria in water bodies are related with the transmission of conjugative plasmids, which additionally carry genes of resistant to sulfonamides and aminoglucosides, giving the bacteria multi-resistant patterns (76). This phenomenon was observed in our study, where the most common higher co-resistant rates among the ESBLs-pE identified for ciprofloxacin (74.6%) and SXT (73.2%). However in this study, the most active drugs for ESBLs-pE were meropenem (94.4%), nitrofurantoin (88.7%) and piperacillin/tazobactam(87.3%)

During sample collection in this study we noticed the selected hospitals had neither WWTP nor wastewater stabilization pond except in TASH nonfunctional waste stabilization pond was observed. Since the present study detected a high proportion of pathogenic, non-susceptible and ESBLs-pE, indicating a higher probability potential risk of microbial pollution of water bodies in the community, hence accelerate spreading of resistant microorganisms into community.

9. Recommendation

The high occurrence of MDR and ESBLs-pE in the present study from hospital effluent may have a severe consequence in the public health. These bacteria can carry several genetic determinants which can be transmitted to another bacterium including pathogens. This indicates a need for a highly committed and consistently hygienic treatment of effluent (implementation of final disinfection procedure to minimize the microbial burden) at each respective wards and units to inhibit the transmission of the antibiotic resistant to another enteric pathogenic bacterium. Since all selected hospitals for this study purpose were hadn't any either wastewater treatment plant or functional wastewater stabilization pond, therefore every concern bodies should take urgent measures to minimize the devastating outcome from the discharge of hospital wastewaters into the community drainages without getting appropriate treatment. We suggest additional studies to take place on molecular epidemiology of ESBLs producing Enterobacteriaceae and their effects on patient recovery and health care burden in Addis Ababa, Ethiopia.

10. Referrence

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Annexes

Annex 1: Sample and data Collection Format

Hospital wastewater sample collection format

Name of hospital:_____

Date of collection: _____

Collection time: _____

Round of sampling: _____

Name of sampling site: _____

Sample number: _____

Method of waste disposal: _____

Infection prevention practice: _____

Sampled by: _____

Signature: _____

Annex 2: Preparation of MacConkey Agar

MacConkey (MAC) agars are slightly selective and differential plating media used for isolation and detection of gram-negative organisms. The medium is prepared from ready to use dehydrated powder, available from the most supplier of it. MA contains are peptone, lactose, bile salts, and crystal violet sodium chloride, methyl red and agar. Its content serve as; Peptone as a nutrient, lactose as a fermentable sugar, methyl red as indicator and crystal violet and bile salts as inhibit of gram-positive organisms while allowing gram-negative organisms to grow.

Procedure

- The medium is usually prepared at a concentration of 5.2g for 100 ml distilled water

1. Weigh the MAC agar according to the instructions given on the label of the dehydrated powder.
2. Suspend the powder in distilled water.
3. Sterilize by autoclave at 121 oc for 15 minutes.
4. When the medium has cooled to 50-55oC, gently mix and aseptically pour into sterile petri dishes inside the BSC to minimize contamination.
5. Then allow the medium to cool at room temperature to remove excess moisture.
6. Label plates with media name and preparation date.
7. Wrap plates in plastic bags, 10 plates per bag to prevent loss of moisture and store at 2-8oc.
    - ✓ Shelf life: up to 8 weeks providing there is no change in the appearance of the medium to suggest contamination or alteration of PH.
    - ✓ PH of medium should be within the range of 7.2-7.6 at room temperature.

- **Quality Control**: Test prepared media for sterility, ability to support growth, and ability to produce appropriate biochemical reactions.
- **Interpretation of Results**
    - ✓ Lactose fermenting organisms grow as pink to brick-red colonies with or without a zone of precipitated bile. The bile precipitate is due to a local pH drop around the colony due to lactose fermentation
    - ✓ Lactose no fermenting organisms grow as colorless or clear colonies. When nonlactose fermenters grow in proximity to coliform colonies, the surrounding medium appears as cleared areas.

Annex 3: Biochemical test procedures

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

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

C. **Triple Sugar Iron (TSI) Agar Slant:** Using a sterile inoculating sterile loop, stab the butt of the TSI slant twice then streak back and forth along the surface of the agar with the organism. Incubate at 37^{O}C for 18 to 24 h.

Result and interpretation

- ✓ If acidic slant /acidic butt (A/A reaction/yellow-yellow): All the sugars (glucose, lactose, and sucrose) are fermented. This is characteristic of lactose fermenting coliforms. Example; E. coli , Klebsiella spp.
- ✓ If alkaline slant /alkaline butt (K/K reaction/red-red): This shows no carbohydrate fermentation. This indicates that the bacteria are non-fermented. Example: Pseudomonas aeruginosa
- ✓ If alkaline slant /acidic butt (K/A reaction/red-yellow): Glucose is fermented. Lactose and sucrose is not fermented. It indicates that the organism is a non -lactose fermenter. Example: Shigella.
- ✓ If alkaline slant /acidic butt /black precipitate of H2S (K/A reaction/red-yellow and positive for H2S): Glucose is fermented. Lactose and sucrose are not fermented. This is characteristic of non -lactose fermenting, hydrogen sulfide producing bacteria. Example: Salmonella spp.
- ✓ Generally the presence of yellow colour, black precipitate (butt) and splits or cracks with air bubbles indicates carbohydrate fermentation, hydrogen sulfide production and gas production respectively.

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

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

F. **Lysine decarboxylase:** Decarboxylation of lysine can be detected by culturing bacteria in a medium containing the desired amino acid, glucose, and a pH indicator bromo cresol purple. The acids produced by the bacteria from the fermentation of glucose will initially lower the pH of the medium and cause the pH indicator to change from purple to yellow. The acid pH activates the enzyme that causes decarboxylation of lysine to amines and the subsequent neutralization of the medium. This results in another color change from yellow back to purple. Bacteria that decarboxylate lysine turn the medium purple. In addition bacteria that produce H_2S appear as black colonies.

G. **Oxidase test**: A piece of filter paper is soaked with a few drops of oxidase reagent(tetramethyl p-phenylenediamine hydrochloride or dimethyl-p- phenylenediamine dihydrochloride). A colony of the test organism is then smeared on the filter paper. Alternatively an oxidase reagent strip can be used. When the organism is oxidase-producing, the phenylene diamine in the reagent will be oxidized to a deep purple colour.

Annex 4: Preparation of Mueller Hinton Agar

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

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

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

Annex 5: Procedure for Performing the Disk Diffusion Test

I. Direct Colony Suspension Preparation

Using a sterile wire loop, touch 3-5 well-isolated colonies of similar appearance from an overnight incubated agar plate (use a non-selective medium, such as nutrient agar) and emulsify in 3-4 ml of sterile physiological saline or nutrient broth to obtain 0.5 McFarland turbidity (1.5 x 10^8 CFU/ ml of the bacteria). For visual comparison, look through the suspension in transmitted light against a white background with contrasting black stripes.

II. Inoculating Test Plates

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

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

III. Antimicrobial Discs Application procedure

Application of antimicrobial discs to an agar plate should be done within 15 minutes of inoculation of plates. The selected antimicrobial discs will be dispensed evenly onto the agar plate with the help of a forceps/sterile needle/surgical blade. Flame the tips

of the applicator intermittently. Each disc must be pressed down to ensure complete contact with the agar surface.

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

IV. Interpretation and Reporting of AST Results

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

Annex 6: Different data collection forms

1) Hospital wastewater collection form

Name of hospital: _____

Date of collection: _____

Collection time: _____

Round of collection: _____

Sampling unit/site: _____

Sample number: _____

sampling unit GPS: N _____ E _____

2) Culture and Isolate Identification Form

A. On MacConkey Agar

- Growth: ☐ No growth ☐

B. If there is growth;

- Colony Characteristics: _____
- Lactose fermentation

LF: ▭ Late LF ☐ NLF ☐

C. Biochemical reactions

Identification of ESBLs producing Enterobacteriacaeinvolves the use of biochemical screening media like; oxidase, indole, urea, motility, Lysine decarboxylase, citrate utilization and triple sugar iron tests

Lab. ID		**Biochemical reactions:**								
		Ox	**urea**	**Ind**	**mot**	**LDC**	**Cit**	**TSI**		
								KK/AA	**gas**	**H_2S**
Result	**Positive**									
	Negative									
Finally identified organism:……										

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

3) ESBLs Producing Screening Form

Name of presumptive ESBL producer Organism:_____

Type of disk	Disk diffusion Criteria for ESBLs Test ZID in mm	Zone of inhibition in mm	Interpretation		Remarks
			Non-susceptible	Susceptible	
ceftazidime (30µg)	>22 mm				
cefotaxime (30µg	>27 mm				

4) Suspicious ESBLs Producing Isolate Confirmation Form

_____

Name of presumptive ESBL producer Organism:

Type of disk	Zone of inhibition(ZID) in mm	Difference ZID in mm	Confirmed ESBLs producer		Remarks
			Yes	No	
Ceftazidime (30µg)					
Ceftazidime-Clavulanic Acid (30/10µg),					
Cefotaxime (30µg					
Cefotaxime-Clavulanic Acid (30/10µg)					

5) Anti-microbial Susceptibility Pattern Results Registration Form

Name of ESBL producer Organism: _____

Sr. No	Antimicrobial disk	Interpretation of AST (mm)			Results ZID (mm)	AST finding			Comment
		R	I	S		R	I	S	
1	Nitrofurantoin	≤ 14	15-16	≥ 17					
2	Amoxicillin-clavulanic acid (20/10µg)	≤ 13	14-17	≥ 18					
3	Cefepime(30µg	≤ 19	SDD1 9-24	≥ 25					
4	Cefotaxime (30µg)	≤ 22	23-25	≥ 26					
5	Cefoxitin (30µg)								
6	Ceftazidime (30µg)	≤ 17	18-20	≥ 21					
7	Chloramphenicol (30µg)	≤ 12	13-17	≥ 18					
8	Ciprofloxacin (5 ug)	≤ 15	16-20	≥ 21					
9	Tazobactam+piperacillin	≤ 12	13-14	≥ 15					
10	Meropenem (10µg)	≤ 19	20-22	≥ 23					
11	Tobramycin (10µg)	≤ 12	13-16	≥ 17					
12	Trimethoprim/sulphamet oxazole (1.25/23.75 µg),	≤ 10	11-15	≥ 16					

Keys: AST= Antibiotic Sensitivity Tests, R=Resistance, I=Intermediate, S=Susceptible, ZID=Zone of Inhibition in Diameter

Annex 7: Dummy tables

Table 1: Magnitude & percentage of presumptive & confirmed ESBLs producing*Enterobacteriacae*from Effluent wastewater at selected governmental hospitals of Addis Abeba, Ethiopia, 2020.

Name of hospital	Isolates n(%)											
	A			B			C			D		
	Iso **n(%)**	pES **n(%)**	cES **n(%)**	Iso **n(%)**	pES**n (%)**	cES**n (%)**	isol**n (%)**	pES**n (%)**	cES**n (%)**	Iso **n(%)**	pES **n(%)**	Ces **n(%)**
TASH												
SPHMMC												
ALERT												
Y12HMC												
MIIRH												

Keys.pES= presumptive ESBLs producing isolate, cES=confirmed ESBLs producing isolate, TASH= Tikur Ambesa Specialized Hospital, SPHMMC= St' Paul's Hospital Millennium Medical College, ALERT=Alert hospital, Y12HMC= Yekatit 12 hospital medical college, MIIRH= Menilik II referral hospital

Table 2:Antimicrobial non-susceptible pattern of *ESBL producing Enterobacteriacae*in Effluent wastewater of different selected governmental hospitals of Addis Abeba, Ethiopia, 2020.

HOSPITAL	Isolate	Antibiotics used											
		TOB	AMC	CFP	CTX	CXT	CAZ	CHL	CPR	F	MER	TZP	STX
TASH	A												
	B												
	C												
	D												
SPHMMC	A												
	B												
	C												
	D												
ALERT	A												
	B												
	C												
	D												
Y12HMC	A												
	B												
	C												
	D												
MIIRH	A												
	B												
	C												
	D												

Note: TOB-Tobramycin, AMC-Amoxicillin-Calvulanic acid, CFP-Cefepime, CTX-Cefotaxime, CXT-Cefoxitin, , CAZ-Ceftazidime, CHL- Chloramphenicol, CPR-Ciprofloxacin, F-Nitrofurantoin, MER-Meropenem, TZP-Tazobactam+piperacillin, SXT-Sulfamethoxazole-trimethoprim,

TASH= Tikur Ambesa Specialized Hospital, PH= Paul's Hospital Millennium Medical College, ALERT=Alert hospital, Y12HMC= Yekatit 12 hospital, MIIRH= Menilik II hospital,

Table 3: Multi-drug non-susceptible level of ESBL producing Enterobacteriacaeisolate in Effluent wastewaterof five governmental hospitals, Addis Ababa, Ethiopia from April 1 to May 31, 2020

Hospital	Spp.	Isolates (No.)	Level of antibiotics resistance								TOTAL MDR (≥R3)
			R0	R1	R2	R3	R4	R5	R6	R7	
TASH	A										
	B										
SPHMMC	A										
	B										
ALERT	A										
	B										
Y12HMC	A										
	B										
MIIRH	A										
	B										

KEYS: R0=non-susceptible to no antibiotics, R1-R7= non-susceptible to 1, 2, 3, 4, 5, 6, and 7 antibiotics; ≥R3=non-susceptible to 3 or more antibiotics from different classes.

Table 4: Distribution of ESBLs producing Enterobacteriacaewith their MDR level at the five governmental hospitals, Addis Ababa, Ethiopia from April 1 to May 31, 2020

Wastewater Collection sites	**ESBLs producing**				**MDR, of ESBLs producing**			
	A	**B**	**C**	**D**	**A**	**B**	**C**	**D**
TASH								
SPHMMC								
ALERT								
Y12HMC								
MIIRH								
TOTAL								

Declaration

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

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This thesis has been submitted with our approval as advisors.

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