Antimicrobial Susceptibility Profile of Common Species of Enterobacteriaceae in Wastewater Samples from Health Facilities, Abattoir and Downstream Rivers in Addis Ababa, Ethiopia

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Abbreviations/ Acronyms

API……..Analytical Profile Index

AMR……...Antimicrobial resistance

ARB……Antimicrobial resistant bacteria

ARG……Antimicrobial resistant genes

BPW……Buffered peptone water

CLSI……...Clinical and Laboratory Standards Institute

EMB……Eosin methylene blue

HGT…….Horizontal gene transfer

HWW…….. Hospital wastewater

MDR……Multidrug resistant

ESBL…….. Extended spectrum beta-lactamases

EPHI……Ethiopian Public Health Research Institute

DDST……..Double disc synergy test

TTB…….Tetrathionate broth

TSA…….Tryptone soya agar

MCIA…….Modified carbapenem inactivation assay

PBP…….Penicillin binding proteins

UHWW…….Untreated Hospital Wastewater

WWTP……Wastewater treatment plant

XLT-4……..…Xylose Lysine tergitol 4

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Abstract

Spread of antimicrobial resistant bacteria and antimicrobial resistant genes is causing serious difficulties in the treatment of infection diseases worldwide. Monitoring of antimicrobial susceptibility profile of various bacterial pathogens in the health facilities and the environment is crucial for designing proper implementation of strategy to hamper spread of resistant pathogens and genes associated with resistance. The aim of this study was to assess antimicrobial susceptibility profiles of members of Enterobacteriaceae isolated from wastewater of health facilities, abattoir and downstream water bodies. A total of 24 wastewater samples were collected from the Menilik II Referral Hospital, Kebena River, Tikur Anbessa Specialized Hospital, Kality WWTP, Addis Ababa Abattoir and Little Akaki River on two occasions. Isolation of different Enterobacteriaceae was conducted according to standard procedures using selective media. Identification of Enterobacteriaceae was done by using biochemical test and API 20E. Susceptibility of isolates to a panel of 12 antimicrobials was investigated using disc diffusion assay and selected MDR isolates were also tested for production of extended spectrum beta-lactamase and carbapenemase using double disc synergy test and modified carbapenem inactivation assay respectively. A total of 54 bacteria isolates belonging to Enterobacteriaceae family were identified: *E. coli* (32%), *Salmonella* spp. (23%), *Klebsiella pneumoniae* (15%), *Enterobacter aerogenes* (11%), *Citrobacter* spp. (7%), *Klebsiella oxytoca* (6%) and *Enterobacter cloacae* (6%), respectively. The highest AMR index of 1 was recorded for *Citrobacter* isolate from TASH wastewater followed by 0.91 for *E. coli* from the same source. All isolates were resistant to 2 or more antimicrobial tested. Multi drug resistance (MDR) to several antimicrobials was recorded, particularly in isolates obtained from hospital water samples and it was more common in *Citrobacter* and *E. coli* isolates. Extended spectrum beta-lactamase

(ESBL) production was detected in 27.3% of MDR isolates all of which were obtained from hospital effluents, whereas none of the isolates were carbapenemase producers. This study showed the abundance of antimicrobial resistant Enterobacteriaceae in wastewater from Hospital, Abattoir and downstream water bodies. Moreover, hospital effluents contain highest fraction of antimicrobial resistant bacteria which are released to receiving water bodies, posing public health threat.

Keywords: *Antimicrobial resistance,* Enterobacteriaceae, *Antimicrobial resistance index, wastewater*

1. Introduction

Antimicrobials are chemicals that kill or inhibit the growth of susceptible microorganisms and some are produced naturally by microbes but many are synthetic. They are used extensively to prevent or to treat microbial infections in human and veterinary medicine (Jury *et al.,* 2010). Large amounts of these compounds are released into municipal wastewater due to excessive consumption and disposal of unused antimicrobials. The uncontrolled and overuse of antimicrobials by human and animal has led to the development of emergence of antimicrobial resistant bacteria compromising the effectiveness of antimicrobial therapy (Mozaz *et al.,* 2014).

Antimicrobial resistant bacteria (ARB) and antimicrobial resistant genes (ARGs) cause serious problems in the treatment of infectious diseases (Samadi *et al*., 2015). Infection by antimicrobial resistant bacterial pathogens becomes a global health care concern in the 21st century not only due to their severity but also they require more complex treatment and expensive diagnostic tests (WHO, 2014; Alanis, 2005). Antimicrobial resistance (AMR) is a serious global threat to human health. While some resistant bacteria are found naturally in the environment resistant pathogens and commensal organisms found in hospital wastewater are released into the environment in several ways, contributing to a web of resistance that includes humans, animals and the environm ent (Bolaji *et al*., 2011). Microbial communities in the hospital environment are usually resistant to several antimicrobials, because of frequent use of various antimicrobials in the hospitals. Wastewater from these hospitals can therefore serve as a source of both antimicrobial resistant bacteria and antimicrobial resistance genes (ARGs), which may be released into the environment ,through sewage systems or directly to the water bodies downstream contaminating the environm ent (Mozaz *et al*., 2014).

In addition, depending on the location, different environments have different resistance profiles, such as the aquatic environment, which can act as reservoirs for AMR, particularly since they are typically endpoints of agriculture runoff and wastewater treatment plant discharge (Williams *et al*., 2016). Sewage systems collect wastewater not only from domestic origin but also from abatt oir and hospitals sources. Moreover, antimicrobial resistance genes (ARGs) carried by bacterial contaminants can be transferred to other bacterial populations including pathogenic bacteria that are found in hospital wastewater (Quynh *et al*., 2017).

In Ethiopia, wastewater from some of the health facilities including hospitals is directly discharg ed to the Rivers (personal observation). These contaminated rivers are usually used for irrigation purpose by small scale farmers growing vegetables. This may cause significant public health concern as a result of infection with drug resistant pathogenic bacteria (to the farmers themselves and consumers of the vegetables) in addition to other toxic chemicals.

Similarly, wastewater from abattoirs can also be contaminated with various bacterial pathogens and commensally organisms resistant to various antimicrobials. Different types of farm animals slaughtered in the abattoir can be infected with a wide range of zoonotic pathogens. Moreover, an abattoir is a usual facility designed and licensed for receiving, holding, slaughtering and inspecting meat animals and meat products before release to the public (Adesoji *et al.*, 2016). Animals coming to abattoir might be exposed to various antimicrobials while in the farm for treatment and prevention of diseases as well as for growth promotion (Hong *et al.*, 2013). In addition, over use and misuse of antimicrobials in food producing and companion animals are commonly practiced leading to selection pressure favoring the development and spread of antimi crobial resistant bacteria communities (Beyene *et al.,* 2016). Therefore, there is high chance of occurrence of multidrug resistant pathogenic and commensal bacterial communities in the

wastewater from abattoir which can contaminate downstream water bodies unless it is properly treated.

Many studies in agricultural settings have reported AMR to be a common problem (Agga *et al.,* 2015). In addition to transfer of resistant pathogens to human through consumption of meat products from animals infected by resistant bacteria through food chain, the resistant bacteria and resistance genes can be disseminated to the environment through abattoir wastewater posing risk of contaminating downstream water bodies and the environment. It is important to investigate the level of contamination and antimicrobial susceptibility of bacteria in animal wastewaters for the sake of health of animals and food safety concern for human consumption (Atieno *et al.,* 2013). Abattoir consume large amount of water resource for washing of carcasses after hide removal from cattle, goats, sheep. These activities result in generation of large amount of wastewater along with other by products including blood and intestines, bones, urine and feces, soft tissue removed during trimming and cutting, and cleaning compounds (Amenu, 2014)**.**

Untreated slaughter house wastewater contains a mixture of fats, proteins and fibers that are capable of contaminating the rivers and sewage systems. Still it can be very hazardous to public health in respect to the waste it generates (Abrha and Tenalem, 2015). Water borne zoonosis is a serious problem in developing countries because of the lack of water treatment facilities and useof untreated wastewater (Svanstrom, 2014). Abattoirs in most developing countries have unh ygienic environments that promote the growth of pathogenic microorganisms (Onuoha *et al*., 2016).

Ethiopia has the largest livestock population in Africa. National economy is significantly depend ent on contribution of the livestock sector. Different butcher houses bring their own animals (cattle, goat and sheep) bought from different market places in Addis Ababa and outside Addis

Ababa to abattoir for slaughter. Although majority of these animals are expected to come origina lly from small holder livestock keepers in rural Ethiopia, where frequent use of antimicrobials is not practiced, some of the animals may come from feedlots where antimicrobials are used freque ntly creating selection pressure on susceptible bacterial communities. In addition, some of the livestock traders treat their animals with broad spectrum antimicrobials before transportation to protect animals from stress associated infections, leading to increased risk of release of antimicrobial resistant bacteria in the Abattoir environment and wastewater from such facilities. Recent study in Ethiopia showed significantly high residue level of tetracycline in slaughtered beef cattle from three slaughter houses in central Ethiopia (Bedada *et al.*, 2012).

Untreated effluent of Addis Ababa abattoir from hundreds of animals slaughtered daily flows in to Little Akaki River directly without any waste treatment (Gudeta *et al.*, 2012). This effluent in downstream rivers and nearby residential areas may serve as reservoir of antimicrobial resistant bacteria and resistance genes posing risk to human and livestock population in the area. Recent study in Ethiopia showed that rate of occurrence of non-typhoid *Salmonella* among diarrheic human patients attending primary health centers was significantly higher in those consuming raw vegetables than those who did not (Eguale *et al.*, 2015).

Most of these isolates were resistant to several drugs. Contamination may occur when harvesting the vegetables or washing ready to eat vegetables with untreated wastewater. In Ethiopia, particularly, in Addis Ababa there is no data concerning resistance profiles of microorganisms isolated from hospitals and abattoir wastewater and downstream water bodies. This study is therefore designed to generate original data on occurrence and antimicrobial susceptibility profile of common Enterobacteriaceae members in wastewater originating from hospitals and an abattoir as well as downstream water bodies in Addis Ababa, Ethiopia.

2. Objectives of the study

1.2.1. General objective

The general objective of this study was to assess the level of antimicrobial resistance in selected members of Enterobacteriaceae isolated from wastewater of two referral hospitals, an abattoir, receiving WWTP in Kality and Rivers in Addis Ababa.

1.2.2. Specific objectives

- To isolate members of the family Enterobacteriaceae from wastewater of Tikur Anbessa Specialized Hospital, Menilik II Referral Hospital, Addis Ababa Abattoir, receiving Rivers of Kebena, Little Akaki and Kality WWTP.
- To identify members of the family Enterobacteriaceae from wastewater of Tikur Anbessa Specialized Hospital, Menilik II Referral Hospital and Addis Ababa Abattoir.
- To evaluate the antimicrobial susceptibility of the identified members of Enterobacteriace ae to common antimicrobials.
- To determine extended spectrum beta lactamase and carbapenemase production in isolate s resistant to second and third generation cephalosporins.

Hypothesis

Wastewater from TASH, Menilik II Ref. Hospital, Addis Ababa Abattoir, the receiving WWTP and downstream Rivers are may be contaminated by various species of Enterobacteriaceae exhibiting resistance to different antimicrobials.

2. Literature review

2.1. Sources of antimicrobial resistant bacteria and antimicrobial resistance genes in the environment

Wastewater is any water whose quality has been adversely harmed by anthropogenic influence and this includes liquid waste released from Municipal, industries, hospitals, and agricultural sectors (Kumarathilaka *et al.,* 2015). Different wastewater runoff in developing countries represe nt important source of rising contaminants like ARGs, ARB for the receiving environment as the effluents are discharged to the sewer systems, rivers, lakes, and seas without pretreatment (Devarajan *et al.,* 2016).

Antimicrobials used for the treatment and prevention of bacterial infections are mainly release dunmetabolized into the aquatic environment via wastewater. Sometimes unused therapeutic drugs are released down drains (Kummerer, 2001). Large amount of antimicrobials and disinfectants are used in hospitals for treatment of patients and disinfection purpose, respectively. The biggest number of antimicrobials taken by the patients are partially metabolized and excreted through feces and urine. After use, a lot of these products reach the wastewater, exposing the bacteria that survive in hospital wastewaters to a wide range of drugs that could act as a selective pressure for the development of resistance (Nunez and Moretton, 2007).

Bacteria may acquire resistance by mutation or be intrinsically resistant to antimicrobial agents (Kumar and Varela, 2013). According to Odonkor and Addo, (2010) the primary cause of antimicrobial resistance is genetic mutation in bacteria. Overuse of antimicrobials in both human and veterinary medicine has resulted in the emergence and spread of strains of resistant microorganisms. Bacteria also develop resistance through the acquisition of new genetic material

from other resistant organisms. This is termed horizontal gene transfer, and may occur between strains of the same species or between different bacterial species or genera (McManus, 1997).

A study by Kummerer, (2004) showed that concentration of ampicillin ranged from 20 to 80 mg/L in the effluent of a large German hospital. Concentrations as high as 5 mg/L was found for benzalkonium chloride, quaternary ammonium compound, in the effluent of European hospitals. Antimicrobials have been discharged into wastewater treatment plants (WWTPs) as chemical pollutant for decades from different sources, such as hospitals, abattoirs, pharmaceutical factories and households. Multiple classes of antimicrobials have been widely detected in different WWTPs worldwide (Zhang, 2016). A study conducted in south Ethiopia reported that *Bacillus* spp. and *Salmonella* isolates obtained from Yirgalem Hospital effluent treated with sodium hypochlorite (with 0.5% free chlorine), were resistant to ceftriaxone, tetracycline, and doxycycli ne, whereas those from Hawassa University Referral Hospital effluent were resistant to the above three antimicrobials and gentamicin as well (Fekadu *et al.*, 2015).

2.1.1. Hospital wastewater

Hospital wastewater is sourced from different areas such as hospitalization rooms, surgery rooms, laboratories, administrative units, laundries, kitchens and forms a major discharge of chemicals (Pauwels and Verstraete, 2006). Hospital waste effluents are discharged into the sewer system, usually pretreatment, may contain multidrug resistant (MDR) Enterobacteriaceae, which constitute the most dangerous single risk factor for dissemination of pathogenic and drug resistant organisms to the environment (Anssour *et al.,* 2016).

The selective environments for antimicrobials like hospitals increase the frequency of resistance bacterial genes and genetic elemets such as plasmids (Diab *et al.*, 2008). If the resistant bacteria

are carrying transmissible genes, they move resistant genes to other bacterial community so that infections caused by these bacteria are frequently difficult to treat (Rodney and Costerton, 2002). The contact of this kind of wastewater by ARG with the surrounding environment results in negatively effect on the biological balance of aquatic ecosystems and public health (Kajitvichyanukul and Suntronvipart 2006).

2.1.2. Abattoir wastewater

Abattoir waste involves parts of animal body that are not used for food production and it can also include urine, feces, and carcasses and blood (Svanstrom, 2014). River bodies' contamination by abattoir wastes either directly or indirectly through various processes, might comprise an importa nt environmental and health hazards, promoting the growth of disease causing organisms such as bacteria (Onuoha *et al.,* 2016). Rivers may be polluted by abattoir effluents and create other environmental stresses in the downstream and near residential areas (Abrha and Tenalem, 2015). Different types of farm animals in the livestock sector are capable of carrying a wide range of zoonotic pathogens (Famotemi *et al.,* 2017).

Occurrence of antimicrobial resistance in the environment has also been associated with antibiotic abuse in animal feedlots (Williams *et al.,* 2016). Wastewater generated from the abattoir is characterized by the presence of many pathogenic microorganisms, such as *Salmonella, Escherichia coli* which have public health importance (Onuoha *et al.,* 2016). Livestock have been implicated as a source of human infection with antimicrobial resistant bacteria (Abebe *et al.,* 2014).

Fecal contamination has been the main source of contamination of undercooked beef with *E. coli* which can be source of infection for human (De *et al.,* 2015). Antimicrobial resistant *Salmonella*

are increasing due to the use of antimicrobial agents in food animals at curative level or prophyla ctic doses which may contribute to on-farm selection of antimicrobial resistant strains which markedly increases the human health risks associated with consumption of contaminated meat products and has emerged as a global problem (Addis *et al.,* 2011).

2.2. Antimicrobial resistance (AMR) in selected Enterobacteriaceae

Resistance to common bacteria has reached alarming rate in many parts of the world according to WHO global report on AMR (WHO, 2014). Below is a brief description of status of antimicro bial resistance in selected gram negative bacterial species such as *Escherichia coli*, *Citrobacter* spp., *Salmonella* spp., *Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter aerogenes* and *Enterobacter cloacae* (Dzidic *et al.,* 2008).

2.2.1. Antimicrobial resistance in *Escherichia coli*

Escherichia coli are member of the normal flora in the human and animal intestine. However, some strains of *E. coli* carrying various virulence genes are pathogenic,capable of causing gastro intestinal infections, urinary tract infections and along with others can cause serious extra intestin al health complications (Galvin *et al*., 2010). Resistance in *E. coli* readily develops either due to chromosomal mutation, which is often the case for fluoroquinolone resistance or by acquisition of mobile genetic elements, which has been the case for broad spectrum penicillins (e.g. ampicillin or amoxicillin) and third-generation cephalosporins (WHO, 2014).

Therefore, subjected to a high selection pressure driven by the antimicrobials to which their hosts are exposed, they may serve as a good indicator to monitor the general level of AMR in a human or livestock population (Strom *et al.,* 2017). *Escherichia coli* isolates resulting from a wide

variety of clinical materials are found to be resistant to several commonly used antimicrobials such as ampicillin (Feleke *et al.*, 2014).

In the Hospital effluent discharge, there have been an increasing number of reports about antimicrobial resistant *E. coli* isolates. Ampicillin resistant *E. coli* was detected in every sample of hospital effluent and was generally present in higher proportions than other antimicrobial resistant *E. coli*, accounting for 100% of the total population in a sample (Galvin *et al*., 2010). A study from Poland showed that 20% of *E. coli* isolates from hospital wastewater collected before 2013 were resistant to co-trimoxazole (Lien *et al.,* 2017). A study conducted in south

Ethiopia showed that *E. coli* isolates were resistant to ceftriaxone, tetracycline, gentamicin, and doxycycline (Fekadu *et al*., 2015). Another study done by Katouli *et al*., (2012) showed that among *E. coli* strains isolated from Untreated Hospital Wastewater (UHWW), the highest resistance was observed against aztreonam, gentamicin, amoxicillin-clavulanic acid, ceftazidime, cefepime ranging from 89% to 79% with the lowest resistance found against ciprofloxacin; norfloxacin, nalixidic acid, nitrofurantoin and chloramphenicol (0% to 12%).

A study by Korzeniewska *et al.,* (2013) indicated that strains of *E. coli* isolated from hospital effluents characterized higher resistance rates. Almost all (95.7%) of the 395 identified *E. coli* strains were shown to be resistant to cefotaxime, a third generation cephalosporin. Resistance to other cephalosporins ranged from 21.1% to 94.7%. Over 61% and 48% of isolates were not sensitive to the standard doses of gentamicin and amikacin, respectively. A study by Tadesse *et al.,* (2012) showed that *E. coli* isolates from animal showed an increasing resistance trend to 11 antimicrobial agents (ampicillin, sulfonamide, tetracycline, cephalothin, trimethoprim/sulfameth oxazole, streptomycin, chloramphenicol, cefoxitin, gentamicin, amoxicillin/clavulanic acid, and

kanamycin), and *E. coli* isolates from human showed an increasing trend in resistance only to ampicillin, sulfonamide, and tetracycline.

2.2.2. Antimicrobial resistance in *Salmonella*

Salmonella is a family of Enterobacteriaceae, rod shaped and Gram negative facultative anaerobe and one of the most common public health problems (Eng *et al*., 2018). It is a disease causing bacterium and represents an important public health problem among the common bacterial food borne pathogens worldwide. *Salmonella* is one of the major causes of diarrhea in humans. The tradition of consumption of raw meat and indiscriminate use of antimicrobials signifies the importance of *Salmonellosis* in Ethiopia (Jelalu *et al.,* 2015). *Salmonella* clinical isolates collected in Addis Ababa three decades ago were usually susceptible to most of the drugs tested. However, in the Northwest Ethiopia from Hospital Wastewater by the year 2011, over 80% of the isolates were resistant to ampicillin, amoxicillin, chloramphenicol and SXT (Feleke *et al.,* 20 14). Resistance to tetracycline among *Salmonella* spp. in Thailand was also 58% in 1998 and 100% in 2003. Antimicrobial agents such as ampicillin and trimethoprim-sulfamethoxazole are used as the traditional first line treatments for *Salmonella* infections (Eng *et al*., 2018).

2.2.3. Antimicrobial resistance in *Klebsiella* Spp.

Members of the genus *Klebsiella* are opportunistic pathogens that are frequently associated with a variety of diseases in animals and humans (Chander *et al.,* 2011; Barati *et al.,* 2016**).** *Klebsiella* spp. is one of the important contamination indicators of water, food and agricultural products to coliforms and wastewater (Maal *et al.,* 2014). Nowadays multidrug resistance in genus *Klebsiell a* has been reported, the main resistance mechanism being production of extended spectrum beta-lactamases (ESBLs) (Flores *et al.,* 2016). Nosocomial infections in humans mainly involve

Klebsiella pneumoniae and *Klebsiella oxytoca* which cause infections of urinary, respiratory tract and mastitis (Chander *et al.,* 2011). Antimicrobial resistance in *Klebsiella pneumoniae* and *Klebsiella oxytoca* is an alarming problem (Maal *et al.,* 2014).

Klebsiella pneumoniae is a Gram negative bacterium belonging to the family Enterobacteriaceae, ubiquitous non-motile aerobic rod that causes a wide variety of hospital-acquired infections and has become important pathogens in nosocomial infections, reported worldwide (Sikarwar and Batra, 2011; Doorduijn *et al*., 2016). *K. pneumoniae* acquires resistance to multiple antibacterial drugs mainly through horizontal transfer of mobile genetic elements such as transpo sons or plasmids. In addition, it carries a resistance gene (chromosomally located beta lactamase) that induces intrinsic resistance to penicillins with an extended spectrum activity, such as ampici llin and amoxicillin also resistance to other widely used drugs like ciprofloxacin has emerged an d spread globally (WHO, 2014).

K. pneumoniae, due to extended spectrum beta-lactamases (ESBL) production, above 50%-60% non-susceptibility to third generation cephalosporins, fluoroquinolones and aminoglycosides has been reported (Venezia *et al*., 2017). A study by Sikarwar and Batra, (2011) showed that *K. pneumoniae* strains from clinical cases were found highly susceptible to quinolones and aminoglycosides, amikacin and gentamicin. At the same time over 60% of strains were found resistant to chloramphenicol and tetracycline. Reports from different clinical samples showed that >80% of *Klebsiella* spp. isolates were resistant to sulfamethoxazole/trimethoprim (SXT) (Feleke *et al.*, 2014).

Klebsiella oxytoca is ubiquitous in the environment and can be cultured from the skin, mucous membranes, oropharynx, as well as a variety of tissues from clinically affected humans and

animals (Darby *et al.*, 2014). *K. oxytoca* is normally acquired from environmental sources and also isolated from different clinical samples mainly from the blood and respiratory secretions, and is gaining clinical significance in immune compromised and debilitated patients admitted in Intensive Critical Care Units (Singh *et al.*, 2016). This bacterium carries a constitutive beta - lactamase that confers low level resistance to penicillis but no significant resistance to other beta lactams (Fenosa *et al.*, 2009). In addition due to production of extended spectrum beta-lactamase (ESBL) there is increasing resistance rate to penicillin and ampicillin. In addition 10-20% of the species in Europe is multi-resistant to broad spectrum cephalosporins like ceftazidim because of carrying plasmid borne beta-lactamases genes and class A chromosomal beta-lactamase (Zedan, 2017). Over production of beta-lactamase enzymes is the major mechanism of resistance which is brought by over expression of constitutive beta-lactamase genes or the presence of multiple copies of beta-lactamase genes (Moradigaravand *et al.*, 2017).

2.2.4. Antimicrobial resistance in *Citrobacter*

The genus *Citrobacter* are Gram negative bacilli, motile, facultative anaerobic belonging to Enterobacteriaceae family. And commonly found in water, soil, food & intestinal tracts of humans, animals and also they are emerging as a nosocomial multidrug resistant pathogen across the world (Avinash *et al.*, 2015**).** Those have been associated with a variety of infectious diseases like hospital-acquired bacteremia, endocarditis, urinary tract infections, neonatal meningitis and brain abscess have been reported (Arens *et al.*, 1997). The high mortality rate associated with *Citrobacter* infections may be due in part to ineffective empirical antimicrobial treatment (Pepperell *et al.*, 2002).

The importance of this species lies in their association with high degree of resistance to common antimicrobial agents used for the treatment of different infections especially extended spectrum

cephalosporins, due to over expression of chromosomal beta lactamases, plasmid and chromoso mal AmpC cephalosporinase, Metallo beta-lactamases, and loss of outer membrane pores (Nayar *et al.,* 2014).

2.2.5. Antimicrobial resistance in *Enterobacter spp*

Enterobacter belongs to the family of Enterobacteriaceae. It is gram negative, facultative anaero bic, rod shaped, and none spore forming bacteria found in the feces of humans, animals and in water (Regli and Pages, 2015). *E. aerogenes* and *E. cloacae* are important opportunistic pathoge ns of humans. These bacteria have been associated with several hospital acquired outbreaks of infections in Europe, particularly in France (Abbas and Radhi, 2016). *Enterobacter cloaca* is widely distributed in the environment and can be a part of the normal flora of human and animal gastrointestinal tracts in particular in immune compromised individuals and also can cause infections in wound, urinary tract, lower respiratory tract (Marti *et al.*, 2017). *E. cloacae* has been reported in many publications particularly over the last 15 years signifying their remarkable ability to up regulate or acquire resistance determinants and making them some of the most worrying microorganisms of the present antimicrobial era (Mezzatesta *et al.,* 2014).

These bacteria have innate resistance to some older antimicrobials and have the tendency to rapidly develop resistance to newer antimicrobials. Some multidrug resistant, strains of *Enterbob acter* species have spilled over into the community, occasionally infecting healthy individuals (Patel and Patel, 2016). These organisms become resistant to beta lactam antimicrobials by producing an extended spectrum beta-lactamase (ESBL) protein, which breaks the beta lactam ring of the antimicrobials and inactivating it (Wilberger *et al.*, 2012).

E. cloacae strain with inducible beta-lactamase is susceptible to third generation cephalosporins and imipenem but resistant to ampicillin and cefoxitin (Yang *et al.,* 1988). A study by Mezzatest a *et al.,* (2014) showed that most isolates of the *E. cloacae* complex are susceptible to fluoroquin olones, trimethoprim/sulfa methoxazole, chloramphenicol, aminoglycosides, tetracyclines, piperacillin–tazobactam and carbapenems, while they are intrinsically resistant to ampicillin, amoxicillin, amoxicillin–clavulanate, first-generation cephalosporins and cefoxitin owing to the production of constitutive AmpC beta-lactamase. *E. cloacae* is currently being considered the third multidrug resistant Enterobacteriaceae species concerned in nosocomial infections after *E. coli* and *K. pneumoniae* due to the diffusion of most frequent extended spectrum beta-lactamases (ESBL) and also due to limited antimicrobial treatment options. *E. cloacae* is great concern with the emergence of carbapenem resistance in some strains of this species (Lee *et al.,* 2012; Regli and Pages, 2015).

Enterobacter aerogenes is also another common agent of hospital acquired infection broadly distributed in the soil, water, dairy products and in the intestine of animals as well as humans. Additionally they are most frequently found in the gastrointestinal tract (Jesumirhewe *et al.,* 2014). This species has become a significant emerging multidrug-resistant (MDR) pathogen worldwide that is responsible for nosocomial infections, including respiratory and urinary tract infections, bacteremia, sepsis, and meningitis (Diene *et al.,* 2012).

2.3. Mechanism of action of selected antimicrobials in Enterobacteriaceae

Antimicrobial agents have diverse modes of action due to the nature of their structure and amount of affinity to certain target sites within bacterial cells. And those that act selectively on critical microbial functions with minimal effects or without affecting host function. Antimicrobia l agents are described as either bacteriostatic or bactericidal. Bacteriostatic antimicrobial agents

inhibit only the growth or multiplication of the bacteria giving the immune system of the host time to clear them from the system. Bactericidal antimicrobial agents kill the bacteria and therefore with or without a competent immune system of the host the bacteria will be dead (Tenover, 2006). Well known mechanisms include inhibition of cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, inhibition of cell membrane function and inhibiting the synthesis of essential metabolites (Dzidic *et al.,* 2008).

2.3.1. Inhibition of cell wall synthesis

Beta lactam antibiotics such as penicillins and cephalosporins function by disrupting how the peptidoglycan molecules connect together to form the cell wall (McDermott *et al.,* 2003; Dzidic *et al*., 2008). Beta lactams are a large class of antimicrobials that have a beta lactam ring in their structure and they are the most widely used antimicrobials in human and veterinary medicine. Beta lactams include penicillin derivatives, cephalosporins, monobactams, and carbapenems.

Glycopeptides such as vancomycin interfere with cell wall synthesis of Gram positive bacteria, but do so by binding to the terminal D-alanine residues of the nascent peptidoglycan chain, thereby preventing the cross-linking steps required for stable cell wall synthesis (Tenover, 2006).

2.3.2. Inhibition of protein synthesis

Several classes of antimicrobial agents such as macrolides, aminoglycosides, tetracyclines, chlor amphenicol and streptogramins act by inhibiting bacterial protein synthesis (ribosome function). Bacterial ribosomes vary in structure from their counter parts in eukaryotic cells. Antimicrobial agents take advantage of these differences to selectively inhibit bacterial growth (Liwa and Jaka, 2015; Tenover, 2006).

2.3.3. Inhibition of nucleic acid synthesis

Antimicrobials like (fluoro) quinolones and rifamycins known to interfere with nucleic acid bios ynthesis. Fluoroquinolones are potent, broad-spectrum antibiotics used to treat infections caused by wide range of Gram-positive and Gram-negative pathogenic bacteria. Fluoroquinolones differ from quinolones by the replacement of the eighth carbon atom of the backbone with a nitrogen atom and the addition of a fluorine atom at the sixth position, giving them more potent antibiotic action and a broader spectrum of activity. Fluoroquinolones target type II topoisomerase (DNA gyrase) mainly in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria. DNA gyrase contains two subunits *gyrA* and *gyrB* whose function is to relax the supercoiled DNA ahead of DNA replication fork by creating negative super coiling. Topoisomerase IV contains *parC* and *parE* subunits with the function of decatinating (unlinking) replicated double stranded DNA (Paterson and Bonomo, 2005). Fluroquinolones inhibit this activity by binding to the active sites of these enzymes leading to inhibition of DNA replication and cell death during replication and causing double strand breaks (Dzidic *et al.,* 2008). Rifampicins inhibit DNA dependent transcription by binding with high affinity to the beta subunit (encoded by *rpoB*) of a DNA-bound and actively transcribing RNA polymerase.

2.3.4. Inhibition of cell membrane function

Antimicrobials that work by inhibiting bacterial cell membrane synthesis such as polymyxins, attack the cytoplasmic membrane of Gram-positive and Gram-negative bacteria and the outer membrane of Gram-negative bacteria. They bind to phospholipids in the cytoplasmic membrane, causing loss of membrane integrity, leakage of cytoplasmic contents and finally cell death (Bock stael and Aerschot, 2009). The cyclic lipopeptide daptomycin apparently inserts its lipid tail into

the bacterial cell membrane causing membrane depolarization and eventual death of the bacterium (Tenover, 2006).

2.3.5. Inhibition of microbial metabolic pathways

Those antimicrobial drugs such as sulfonamides and trimethoprim (TMP) block the pathway for folic acid synthesis, which ultimately inhibits DNA synthesis. The common antibacterial drug combination of TMP a folic acid analogue plus sulfamethoxazole (SXT) inhibits the enzymatic pathway for bacterial folate synthesis so both of these antibiotics disrupt the production of a metabolic product the microorganism needs and they are both bacteriostatic (Liwa and Jaka., 2015, Tenover, 2006).

3.4. Antimicrobial resistance mechanisms

3.4.1. Resistance by influx–efflux systems

The intrinsic capacity of bacterial cells to control the entry and exit of small molecules is more marked in Gram-negative bacteria, in which the outer membrane provides an effective barrier and constitutes a first line defense against antimicrobial challenge (Eren *et al*., 2013). It has been estimated that *Escherichia coli* has a large number of genes (about 600) encoding small molecule transport proteins (Riley *et al.,* 2006). Activation of efflux pumps has been observed to be a major means of antibiotic resistance in many bacteria and with many antibiotics. The efflux systems pump out the antibiotics that have managed to enter into the cells, thereby preventing their intracellular accumulation and effect on microorganisms (Li and Nikaido, 2004; Piddock, 2006).

Bacterial drug efflux pumps have been categorized into five families, i.e., the ATP-binding cassette (ABC) super family, the major facilitator super family (MFS), the multidrug and toxic

compound extrusion (MATE) family, the small multidrug resistance (SMR) family (a subgroup of the drug/metabolite transporter super family) and the resistance-nodulation-division (RND) super family (Odonkor and Addo, 2010). In particular, drug exporters belonging to RND family play a key role in clinically relevant resistance in Gram-negative bacteria (Li and Nikaido, 2004).

The most well studied efflux system is AcrAB/TolC system in *E. coli*. This system comprises of an inner membrane protein, Acr B, and an outer membrane protein, TolC, linked by a peri plasmic protein, AcrA. When activated, the linker protein folds on itself, bringing the AcrB and Tol C proteins in close contact, thus providing an exit path from the inside to the outside of the cell. Antibiotics are pumped out through this channel (Beceiro *et al*., 2013). In antibiotic-sensitive cells, the AcrAB/TolC system is under repression by the product of *acrR* gene. A mutation in *acrR*, causing change in arg45cys, activates expression of the system and consequent drug efflux (Alanis, 2005).

i. Resistance by chemical alteration of antibiotics

The classical example of this mode of resistance is the action of beta-lactamase enzymes which cleave the beta lactam ring of beta lactam antibiotics (penicillin and cephalosporin). The enzymes discovered early (TEM-1, TEM-2 and SHV-1 beta-lactamases) were capable of inactivating penicillin but not cephalosporin. But subsequent variants with a variety of amino acid substitutions in and around their active sites were identified in many resistant organisms. These have been collectively called extended spectrum beta-lactamases (ESBLs) and also act on later generation beta lactam antibiotics (Munita and Arias, 2016). The early beta-lactamases were sensitive to inhibitors such as clavulanic acid and sub lactam. The most potent and relatively newer group of drugs among the members of the beta lactam family are the carbapenems (imipenem, meropenem, panipenem, ertapenem), which have a broad antibacterial spectrum,

including ESBL-producing pathogens, and are used in the therapy of infections that are not controlled by other members of the family (WHO, 2014). However, carbapenemases, contributin g to resistance of the most carbapenem antibiotic resistance have also been discovered (Crouch *et al*., 2015). There is continuous discovery of newer families of ESBLs causing much concern in public health.

ii. Antibiotic resistance due to target alterations

The common example to this mechanism is the alteration in structure of penicillin binding proteins (PBPs) in penicillin resistance. Mutational changes in PBPs such as reduction in the affinity of PBPs to penicillin and over expression of endogenous low-affinity PBPs encoding genes are known to confer resistance (Elbossaty, 2017).

Similarly, resistance to fluoroquinolone occurs through mutations in the genes coding for the subunits of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*). Eight amino acid substitutions in *gyrA* and two in *gyrB* have been shown to cause fluoroquinolone resistance. Likewise, three sites of mutations in *parC* and one in *parE* have been identified (Dzidic *et al.*, 2008).

3. Materials and methods

3.1. Study design

Cross-sectional study design was employed at two different months of the year 2017-2018 to determine occurrence of common antimicrobial resistant Enterobacteriaceae in the wastewater from two hospitals and the Addis Ababa abattoir facility. The downstream rivers contaminated with wastewater from these facilities in Addis Ababa city were studied.

3.2. Description of study area

The study was conducted in Addis Ababa, capital city of Ethiopia. It is located at 9.02 latitude and 38.75 longitude situated at elevation of 2405 meters above sea level. Addis Ababa has a population of 3,384,569 according to the 2007 population census making it the largest city in Ethiopia. Tikur Anbessa Specialized Referral Hospital (TASH), Menilik II Referral Hospital and Addis Ababa Abattoir facility were selected sites for this study. Kebena River is included as study site because it receives wastewater discharges from Menilik II Referral Hospital. Sewage from Tikur Anbessa Hospital is directly connected to the main sewage line feeding the Kality wastewater treatment plant (Kality WWTP) before getting access to treatment plant. Little Akaki River that is contaminated by wastewater discharged from Addis Ababa Abattoir were also included in the study. These centers were selected based on the fact that their sewer system joins nearby rivers and the existing sewer line to Kality wastewater treatment system. Several small scale vegetable farming is practiced using Kebena and Little Akaki Rivers contaminated with wastewater from Menilik II Referral Hospital and Addis Ababa Abattoir, respectively.

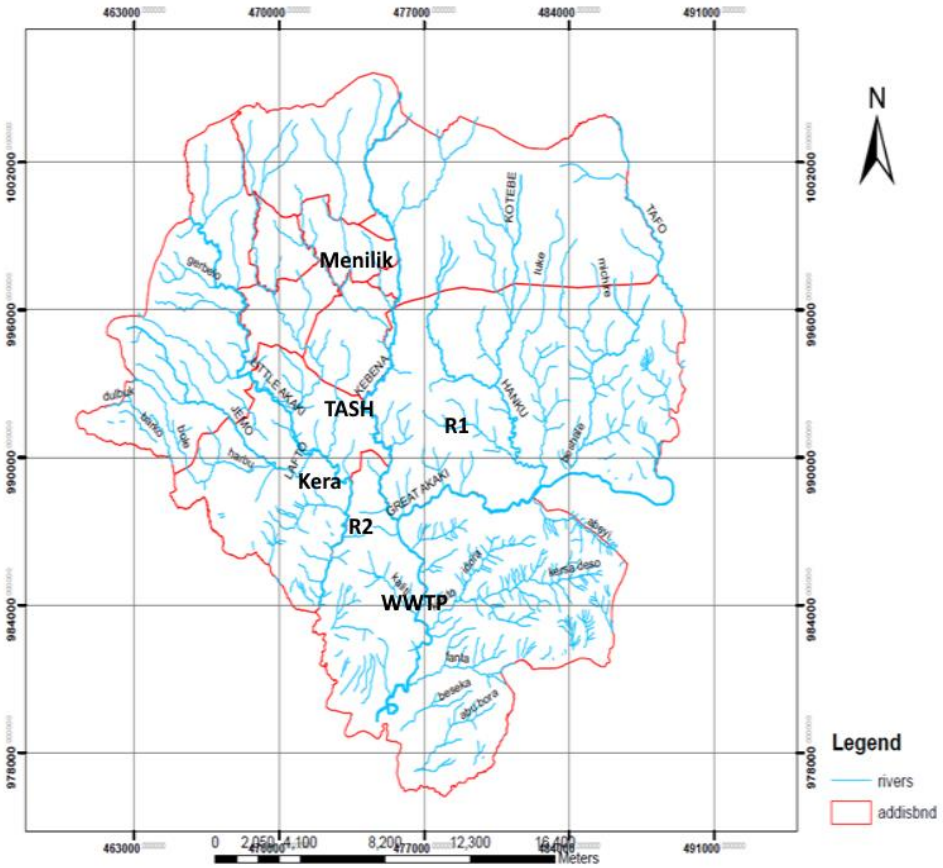

Figure 1. Sampling points from the hospitals, abattoir, rivers and a wastewater treatment plant in Addis Ababa (Source: Yohannes and Elias, 2016).

Description: TASH-Tikur Anbessa Specialized Hospital, Menilik – Menilik II Referral Hospital, Kera – Addis Ababa abattoir, R1 – Kebena River, R2 – Little Akaki River and WWTP – Kality Wastewater treatment plant.

Tikur Anbessa Referral Specialized Hospital (TASH) is located at the center of Addis Ababa and it was established in 1953 E.C. TASH is located at $09^{0}01.265$' N Latitude and $038^{0}45.081$' E Longitude. TASH is largest referral hospital in Addis Ababa and is the main teaching hospital for both clinical and preclinical training and also provides referral services to the community in Addis Ababa as well as other regions of the country. It has several wards Surgical, Pediatrics, Gy necology and Obstetrics, Ophthalmology, Dermatology, Medical, and Labor wards. About 700

outpatients are getting different services per day. The wastewater sources generated from the hos pital includes students' dormitory, toilets, laboratories (Clinical Chemistry, Parasitological, Mole cular, Microbial and Serological Laboratories etc) and from cafeterias flow directly to the main sewage line that joins Kality wastewater treatment plant. Based on personal communication with the hospital communities the hospital does not have functional onsite treatment plants at the time of sampling.

Menilik II Referral Hospital is established in 1910 and it is located at $09^0$02.348' N Latitude and $038^0$46.581' E Longitude in Addis Ababa. The hospital has 29 active specialized case teams with 199 numbers of beds. It has been serving as referral hospital for people with Glaucoma and other chronic eye diseases coming from different regions of the country. It has different outpatient and inpatient departments. It is assumed to be of high rate of waste generation and the wastewater from the Hospital was directly discharged in to Kebena River.

The Addis Ababa Abattoir (Kera Abattoir) is found in central Addis Ababa and located at N $08^0$59.244' and E $038^0$44.793'. It is the largest abattoir in the city providing 85% of the city's meat demand and on average about 400 goats, 600 sheep and 700 cattle are slaughtered daily. According to the information obtained from the organization, Addis Ababa Abattoir uses large amount of water, estimated more than 211,570 liters/day for washing meat and cleaning processing areas. This large amount of water consumption leads to generation a significant amount of wastewater, which is directly discharged in to Little Akaki River without any prior treatment after it travels about 60 meters on land (Abrha *et al.,* 2015).

Little Akaki River (locally known as Kera River) is located at N $08^0$57.635' and E $038^0$44.647'. It receives wastewater from Addis Ababa Abattoir and several small-scale vegetable growing farms are using this river for irrigation purpose.

Kaliti WWTP is located in the southern part of Addis Ababa, located at N $08^0$55.138' and E $038^0$45.456'. The Kaliti wastewater treatment plant was commissioned in 1981 with a design capacity of 7,600 m/day flow and 3,500 kg/day biochemical oxygen demand. Treatment consists of inlet screens and grit chambers, two settling cambers, and two parallel pond systems, each made up of a facultative pond, a maturation pond and two polishing ponds. The wastewater from housing units and TASH connected to the city's sewer system is conveyed to the treatment plant by the sewer network while the sewage from residences and different institutions is transported by vacuum truck (Kassahun and Assefa, 2012).

Kebena River is one of the major stream which flows across Addis Ababa. Menilik II Referral Hospital wastewaters join Kebena River and this river is used by farmers around Peacock Park to grow vegetables. Sample was collected from this river around peacock park, N $09^0$00. 129' and E $038^0$46.652', where it is used for irrigation. Sample from this river was collected at N $08^0$57.635' and E 038^0 44.647' where the river is used widely for irrigation.

3.3. Sample collection and isolation of selected bacteria

3.3.1. Sample collection

A total of 24 wastewater samples were collected from the 6 collection points, on two occasions as shown in table 1. Duplicate samples Wastewater samples from each site were collected using 500ml sterile bottles. The sample was then labeled with date of collection, place of collection

and transported within 3-4 hrs in an ice box to Microbiology laboratory, Aklilu Lemma Institute in Pathobiology for bacterial isolation.

Table 1. Wastewater sampling sites, date of sampling and number of samples collected.

Sampling site	Date of collection		No. of samples*
	First	Second	
Menilik II Referral hospital	November 21/2017	December 2/2017	4
Kebena River	December 11/2017	January 13/2018	4
Tikur Anbessa Specialized Hospital	November 21/2017	December 2/2017	4
Inlet of Kality WWTP	December 11/2017	January 13/2018	4
Addis Ababa abattoir	November 21/2017	December 2/2017	4
Little Akaki river	December 11/2017	January 13/2018	4
Overall Total	**12**	**12**	**24**

*Duplicate samples were collected during each sampling

3.3.2. Isolation of bacterial species

Isolation of members of the family Enterobacteriaceae was conducted according to standard procedures using general and selective media. The selective and differential culture media Eosin methylene blue (EMB) agar was used for the isolation of *Klebsiella* spp, *Escherichia coli*, *Enterobacter* spp and *Citrobacter* spp. Loop full of the sample suspension were inoculated using sterile inoculating loop on pre-dried sterile agar plates of each selective media. The plates were incubated at 37°C for 24 hours.

Salmonella were isolated from wastewater by inoculating samples in to pre-enrichment media, buffered peptone water (BPW) at a ratio of 1:9 and incubated for 24 hours at 37^{o}C. The 100 µl of this suspension were transferred to 10 ml of Rappaport-Vassiliadis enrichment Broth (RVB), and incubated for 24 hours at 37°C. One ml of suspension was transferred to 10 ml of Tetrathionate broth (TTB) and incubated for 24 hours at 42°C. The samples from these two broths were streaked on to Xylose Lysine tergitol 4 (XLT-4) selective media and the plates were incubated at

37°C for 24 hours. Presumptive *Salmonella* colonies were then inoculated into tryptone soya agar (TSA) slant and grown over night at 37^{o}C and kept for further analysis.

3.3.3. Identification of bacterial species using biochemical tests

Distinct presumptive colonies of each suspected bacterial species were picked and confirmed using various biochemical tests as appropriate for each species by growing on agar slants. List of biochemical tests used and typical features for each species is shown in table 2.

Table 2. List of biochemical tests employed to identify typical bacterial species and specific features associated with each species

Species to be identified	Biochemical tests and expected results				
	Urease	**Lysine iron agar(reaction slant/butt)**	**Citrate utilization**	**Triple sugar iron agar**	**Motility**
Salmonella	Negative	Alkaline/alkaline and H_2S production	Positive	Alkaline/acid with gas and H_2S production	Positive
Citrobacter	Positive/ Negative	Alkaline/acid	Positive	Acid/ acid, gas production, +/- H_2S production	Positive
Klebsiella spp	Positive	Alkaline/alkaline	Positive	Acid/Acid, gas production, no H_2S	Negative
E. coli	Negative	Alkaline/acid	Negative	Acid/Acid or alkaline, gas production, no H_2S	Positive
Enterobacter spp	Negative	Alkaline/acid	Positive	Acid/Acid, gas production, no H_2S	Positive

In addition to the conventional biochemical tests, identification of *Citrobacter, Klebsiella,* and *Eterobacter* spp. were confirmed using API 20E kits (Analytical Profile Index), a biochemical panel for identification and differentiation of members of the family Enterobacteriaceae. API20E kits is the plastic strip which holds twenty mini test chambers containing dehydrated media havin g chemically defined compositions for each test and the software APIWEB (Biomérieux, France) was used to interpret the result of the reading after incubation of the organism in each chamber according to the instruction provided by the manufacturer. Isolates were tested for Gram positivity and Gram negativity using 3% KOH. Overnight culture of colonies were picked from agar plates and stirred with drops of KOH reagent on glass slide and then the result was confirmed to gram negative if there is formation of a viscous (Gregerse, 1978).

3.3.4. Investigation of antimicrobial susceptibility of isolated bacterial species

Susceptibility of bacterial isolates to antimicrobials were determined using disc diffusion assay according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013). Briefly, isolates were sub-cultured on tryptic soy agar (Becton, Dickinson and Company, USA) from which 3 to 4 pure colonies were inoculated to a tube containing 5 ml of tryptic soy broth (TSB) (Becton, Dickinson and Company, USA) and mixed gently using sterile inoculating loop. It was then incubated at 37°C for 4-5 hrs. The turbidity of the suspension was then adjusted to the optical density of a McFarland standard of 0.5 using sterile saline to standardize the inoculum size. Sterile cotton swabs were dipped and rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum. It was then inoculated to Mueller Hinton Agar plate (Oxoid, Ltd) by streaking the swab over the entire surface of the plate. The inoculated plates were left at room temperature to dry for 5-10 minutes and antimicrobial discs were dispensed by pressing on the plate with sterile forceps and the plates were inverted and incubated at 37°C overnight. Diameters of the zone of inhibition were measured to the nearest millimeter using a plastic ruler.

The following antimicrobials (Sensi-Discs, Becton, Dickinson and Company, Loveton, USA) and disc potencies were used: Amoxicillin + Clavulanic acid (Amc) (20/10 µg), Ampicillin (Amp) (10 µg), Cefoxitin (Fox) (30 µg), Ceftriaxone (Cro) (30 µg), Cephalothin (Cf) (30 µg), Ciprofloxacin (Cip) (5 µg), Gentamicin(Gm) (10 µg), Streptomycin(S) (10 µg), Sulfisoxazole (G) (1000 µg), Sulfamethoxazole + Trimethoprim (Sxt) (23.75/1.25 µg), Trimethoprim (Trm)(5 µg) and Tetracycline (Te) (30 µg). The interpretation of the categories of susceptible, intermediate or resistant was based on the CLSI guidelines (CLSI, 2013). Isolates were regarded

as multi-drug resistant (MDR) when they were resistant to at least two or more drugs (Magiorakos *et al.,* 2012). *E. coli* ATCC 25922 was used as a quality control organism.

3.3.5. Double-Disc Synergy Test (DDST)

Double-disc synergy test (DDST) was conducted to detect production of extended spectrum beta-lactamase among selected Enterobacteriaceae (n=22) which exhibited intermediate or full resistance to second and third generation cephalosporins (cefoxitin, ceftriaxone) in addition to ampicillin and cephalothin. This test was particularly designed to detect ESBL production in Enterobacteriaceae (Drieux *et al.,* 2008). The test isolates were grown in Muller Hinton Broth for 3-4 hrs and standardized to equal turbidity level of 0.5 Mac Ferland standards. It was swabbed on a surface of Mueller-Hinton agar plate on which 30µg disk of cefotaxime and a disk of amoxicillin–clavulanic acid (containing 20µg/10µg) was placed at a distance of 20 mm center to center (Kaur *et al.,* 2013). The plates were incubated at 37°C overnight and zone of inhibition was measured using ruler. The selected bacterial isolates were considered as positive for the ESBL production when a decreased susceptibility to ceftriaxone is combined with a clear-cut enhancement of the inhibition zone of ceftriaxone in front of the clavulanate-containing disc (CLSI, 2017).

3.3.6. Modified carbapenem inactivation assay

Carbapenemases are broad spectrum beta-lactamases that hydrolyze carbapenems as well as other beta lactams, and are frequently produced by Enterobacteriaceae. Modified carbapenem inactivation assay (MCIA) was used to assess production of carbapenemase in suspected Enterobacteriaceae (CLSI, 2017). Briefly carbapenemase production was tested by using 10µg meropenem disc. Pure culture of the organism's inoculums were prepared by suspending the fresh grown bacteria in sterile Mueller Hinton broth and 10 µg Meropenem disc were immersed

into test tube and incubated for 4 hours at 37°C. A disc which contained Meropenem (10 µg) was placed on the Mueller Hinton agar that was swabbed by *Escherichia coli* ATCC 25922 and the plates were incubated at 37°C for 18-24 hours. Carbapenemase production was considered positive when zone of inhibition of 6–15 mm or presence of colonies within 16–18 mm zone was observed and negative when zone of inhibition is greater than or equal to 19 mm (CLSI, 2017).. If the test isolate does not produce carbapenemase, the meropenem in the disc will not be hydrolyzed and inhibit the growth of meropenem susceptible *E. coli* ATCC 25922.

3.3.7. Quality control

Qualities of data obtained were insured by following standard procedures in each step of the work. The functionality of instruments and quality of media, reagents, and antimicrobial disc was checked by following the manufacturer's direction before employing in the experiment. Reference strains (quality control strains) were obtained from Ethiopian Public Health Institute (EPHI) and employed to control the performance of biochemical tests, disc diffusion test and modified carbapenemase inactivation assay: *Escherichia coli* ATCC 25922, *K. pneumoniae* ATCC BAA-1705 and *K. pneumoniae* ATCC BAA-1706 were used according to CLSI recommendation.

4. Data analysis

Data was entered to Microsoft Excel and descriptive statistics such as percentage were used to analyze the data. Microsoft Excel was also used for generating the graphical presentation.

5. Ethical Approval

This study was submitted to the College of Natural Sciences Institutional Review Board (IRB) for ethical approval. The Approval was executes on the minute no. IRB/031/2018.

6. Results

6.1. Distribution of bacterial genera isolated from the wastewater samples

A total of 54 isolates belonging to different genera of the family Enterobacteriaceae were identified from the six wastewater samples in two rounds. Among all bacterial species examined *E. coli* was isolated from samples from all sites dominantly obtained from Addis Ababa Abattoir and Hospitals, comprising four and five of the total characterized isolates. The second most commonly isolated member of the family Enterobacteriaceae was *Salmonella* spp it was isolated from TASH, Kality WWTP, Addis Ababa Abattoir and Little Akaki River. *Klebsiella pneumoniae* was the third most dominant species it was isolated from Menilik II Referral Hospital, Addis Ababa Abattoir and Little Akaki River. *Enterobacter cloacae* were the fourth dominant species which is obtained from all six sample site. *Enterobacter aerogenes* was isolated from two of the six sites namely Addis Ababa abattoir and Little Akaki River. *Citrobacter* spp. characterized isolates detected from TASH, Little Akaki river and Kality WWTP effluent. *Kelbsiella oxytoca* were obtained from TASH, Menilik II Referral Hospital and Little Akaki river (Table.3).

Table 3. Distribution of members of the family Enterobacteriaceae isolated from hospitals and abattoir wastewater, their receiving Rivers and Kality WWTP

Isolated Bacteria	No						
	TASH	Menilike II Referal hospital	Addis Ababa Abattoir	kebena river	Little Akaki river	Kality WWTP	Total
E. coli	4	3	5	1	3	2	18
K. pneumoniae	-	2	2	2	1	1	8
K. oxytoca	1	1	-	-	1	-	3
Salmonella spp	1	1	2	2	3	3	12
Citrobacter spp	2	-	-	-	1	1	4
Enterobacter aerogenes	-	-	2	-	1	-	3
Enterobacter cloacae	2	1	2	1	2	1	9
Total	10	8	13	6	12	9	54

6.2. Antimicrobial susceptibility of *Citrobacter* Spp. to selected antimicrobials

A total of four *Citrobacter* isolates were obtained from three sites; two of which were from wastewater of Tikur Anbessa Specialized Hospital (TASH) and one each from Little Akaki River

and Kality WWTP. Both isolates collected from TASH were resistant to all antimicrobials tested. A single *Citrobacter* strain isolated from Little Akaki River was resistant only to amoxicillin+clauvlanic acid, tetracycline, ampicillin, cephalothin, ceftriaxone, whereas a single *Citrobacter* strain isolated from Kality WWTP was resistant to amoxicillin+clauvlanic acid, ciprofloxacin, tetracycline, ampicillin, cephalothin, cefoxitin, ceftriaxone, sulfisoxazole (Table 4). *Citrobacter* spp isolated from Kality WWTP was sensitive to gentamycin, sulfamethoxazole/trimetoprim, trimetoprim and resistant to amoxicillin/clavunic acid, ciprofloxa cin, tetracycline, ampicillin, cephalotin, cefoxitin, ceftriaxone and sulfisoxazole. Intermediate resistance was observed to streptomycin. *Citrobacter* isolated from Little Akaki River was sensitive to gentamycin, streptomycin, sulfamethoxazole/trimetoprim, trimetoprim, cefoxitin, s ulfisoxazole and intermediate to ciprofloxacin.

Table 4. Antimicrobial susceptibility profile of *Citrobacter* Spp isolated from effluents of TASH, Kality WWTP and Little Akaki River

	No. of isolates exhibiting resistance			**No.(% resistant of all isolates) (N=4)**
Drug	**TASH (N=2)**	**Little Akaki River (N=1)**	**Kality WWTP (N=1)**	
amoxicillin/clavulanic acid	2	1	1	4(100)
Ciprofloxacin	2	0	1	3(75)
Gentamicin	2	0	0	2(50)
Streptomycin	2	0	0	2(50)
sulfamethoxazole/trimethoprim	2	0	0	2(50)
Trimethoprim	2	0	0	2(50)
Tetracycline	2	1	1	4(100)
Ampicillin	2	1	1	4(100)
Cephalotin	2	1	1	4(100)
Cefoxitin	2	0	1	3(75)
Ceftriaxone	2	1	1	4(100)
Sulfisoxazole	2	0	1	3(75)

6.3. Antimicrobial susceptibility of *Enterobacter* species

Three *Enterobacter aerogenes* isolates were obtained in this study two from Addis Ababa Abattoir, one from Little Akaki River. Interestingly, all the three isolates were susceptible to majority of the antimicrobials examined. Resistance was recorded in all isolates to cephalothin, one of the isolate from Addis Ababa Abattoir and one isolate from Little Akaki River were resistant to cefoxitin while a single isolate from Little Akaki River was resistant to ceftriaxone. Resistance to tetracycline was exhibited only by a single isolate from Little Akaki River (Table 5). *Enterobacter* isolates obtained from Addis Ababa Abattoir were sensitive to gentamycin, strept omycin, sulfamethoxazole/trimetoprim, trimetoprim, tetracycline, ceftriaxone and sulfisoxazole. Among the isolates obtained from Addis Ababa Abattoir, one of the isolates was sensitive while the other exhibited intermediate resistance to streptomycin, cefoxitin. Both of the isolates exhibited intermediate resistance to ciprofloxacin.

Table 5. Antimicrobial resistance profile of *Enterobacter aerogenes* isolated from Addis Ababa Abattoir and Little Akaki River

Drug	No. of isolates exhibiting resistance		No.(% resistant of all isolates) (N=3)
	Addis Ababa Abattoir (N=2)	Little Akaki River (N=1)	
amoxicillin/clavunic acid	0	0	0
Ciprofloxacin	0	0	0
Gentamycin	0	0	0
Streptomycin	0	0	0
sulfamethoxazole/trimethoprim	0	0	0
Trimethoprim	0	0	0
Tetracycline	0	1	1(33.3)
Ampicillin	2	1	3(100)
Cephalotin	2	1	3(100)
Cefoxitin	1	1	2(66.7)
Ceftriaxone	0	1	1(33.3)
Sulfisoxazole	0	0	0

On the other hand, 6 strains of *Enterobacter cloaceae* were isolated in this study 2 from samples of TASH, 1 from Menilik II Referral Hospital, and one isolate each from Kebena River, Little Akaki River and Kality WWTP. Majority of *E. cloaceae* strains isolated in the current study were resistant to most of the beta lactam antimicrobials like ampicillin, cephalothin and even second-generation cephalosporin (cefoxitin) except a single isolate from Little Akaki river. Interestingly, the two isolates obtained from wastewater sample from TASH were resistant to most of antimicrobials tested including ciprofloxacin and ceftriaxone (third generation cephalosp orin) (Table 6).

One of the isolates from TASH was sensitive to gentamycin, trimetoprim and showed intermedia te resistance to streptomycin. The isolate from Menilik II Referral hospital was sensitive to genta mycin, trimetoprim, tetracycline and showed intermediate resistance to ciprofloxacin, streptomyc in, sulfamethoxazole/trimetoprim and ceftriaxone. The isolate from Kebena River showed intermediate resistance to streptomycin and tetracycline. The isolate was sensitive to gentamycin, sulfamethoxazole/trimetoprim, trimetoprim, ceftriaxone, and sulfisoxazole. The isolate from Kality WWTP exhibited intermediate sensitivity to ciprofloxacin and was susceptible to gentamy cin, streptomycin, sulfamethoxazole/trimetoprim, trimetoprim, tetracycline, ceftriaxone and sulfisoxazole.

Table 6. Antimicrobial susceptibility profile of *Enterobacter cloacae* isolated from Tikur Anbessa Specialized hospital (TASH), Menilik II Referral Hospital, Kebena River, Little Akaki River and Kality WWTP

Drug	No. of isolates exhibiting resistance					No.(% resistant of all isolates) (N=6)
	TASH (N=2)	Menilik II Referral hospital (N=1)	Kebena River (N=1)	Little Akaki River (N=1)	Kality WWTP (N=1)	
amoxicillin/clavulanic acid	2	1	1	0	1	5(83.3)
Ciprofloxacin	2	0	1	0	0	3(50)
Gentamycin	1	0	0	0	0	1(16.7)
Streptomycin	1	0	0	0	0	1(16.7)
sulfamethoxazole/trimetoprim	2	0	0	0	0	2(33.3)
Trimethoprim	1	0	0	0	0	1(16.7)
Tetracycline	2	0	0	1	0	3(50)
Ampicillin	2	1	1	1	1	6(100)
Cephalotin	2	1	1	1	1	6(100)
Cefoxitin	2	1	1	0	1	5(83.3)
Ceftriaxone	2	0	0	0	0	2(33.3)
Sulfisoxazole	2	1	0	0	0	3(50)

6.4.Antimicrobial susceptibility of *Escherichia coli* isolates

Antimicrobial susceptibility was tested for a total of 18 *E. coli* isolates collected from all six sampling sites as shown in Table 7. All *E. coli* isolates (n=18) obtained from all sites were 100% resistant to ampicillin and cephalothin, whereas 15(83.3%) of the isolates were resistant to amoxacillin/clavulanic acid, of which all of the isolates from TASH wastewater (n=7) were resistant. The next high rate of resistance (72.2%) was recorded to tetracycline in which a resistance rate of 50%-100% was recorded in isolates from different sources. Resistance to

cefoxitin (2nd generation cephalosporin) was detected in 10(55.6%) of the isolates whereas resistance to ceftriaxone (3rd generation cephalosporin) was detected in 5(27.8%) of the isolates in which three of the resistant isolates were from the two hospital wastewater samples and the two others isolates were from Little Akaki River. The overall rate of resistance to majority of the drugs was high in *E. coli* isolates obtained from the two hospital wastewater samples, followed by isolates from Addis Ababa Abattoir wastewater (Table 7).

Two of the four isolates from TASH were sensitive to sulfamethoxazole/trimetoprim, sulfisoxazo le and one was sensitive to ciprofloxacin, streptomycin, trimetoprim, tetracycline, cefoxitin, ceftr iaxone, sulfisoxazole. One isolate exhibited intermediate resistance to ciprofloxacin, streptomyci n and ceftriaxone.

One of the three *Escherichia coli* isolates from Menilik II Referral hospital wastewater showed intermediate resistance to streptomycin. From Addis Ababa Abattoir wastewater, three isolates showed intermediate resistance to sulfisoxazole; and two isolates showed intermediate resistance to ciprofloxacin, while one isolate showed intermediate resistance to gentamycin, sulfamethoxaz ole/trimetoprim and tetracycline.

The isolate from Kebena River showed intermediate resistance to gentamycin and streptomycin. From Little Akaki River, one of the isolates showed intermediate resistance to Ceftriaxone. One of the two isolates from Kality WWTP showed intermediate resistance to ciprofloxacin, streptom ycin and cefoxitin.

Table 7. Antimicrobial susceptibility profiles of *E. coli* isolates from Tikur Anbessa Specialized hospital (TASH), Menilik II Referral Hospital, Kebena River, Little Akaki River and Kality WWTP

Drug	No. of isolates exhibiting resistance						No.(% resistant of all isolates) (N=18)
	TASH (N=4)	Menilik II Referral hospital (N=3)	Addis Ababa Abattoir (N=5)	Kebena river (N=1)	Little Akaki River (N=3)	Kality WWTP (N=2)	
Amoxicillin/clavulanic acid	4	3	4	0	3	1	15(83.3)
Ciprofloxacin	2	3	0	0	0	0	5(27.8)
Gentamicin	0	0	0	0	0	0	0
Streptomycin	2	2	2	0	1	0	7(38.9)
Sulfamethoxazole/ trimethoprim	2	1	2	0	1	0	6(33.3)
Trimethoprim	3	1	2	0	1	0	7(38.9)
Tetracycline	3	2	3	1	3	1	13(72.2)
Ampicillin	4	3	5	1	3	2	18(100)
Cephalothin	4	3	5	1	3	2	18(100)
Cefoxitin	3	1	4	0	1	1	10(55.6)
Ceftriaxone	2	1	0	0	2	0	5(27.8)
Sulfisoxazole	2	1	2	0	1	0	6(33.3)

6.5. Antimicrobial susceptibility of *Klebsiella* spp.

Among eight *Klebsiella pneumoniae* isolates investigated in the current study, highest resistance rate was recorded against ampicillin and cephalothin (100%) followed by amoxicillin+clavulanic acid (62.5%) and cefoxitin (50%) and tetracycline (37.5%). Resistance to trimetoprim and ceftria xone was detected in 25% of the isolates. Overall occurrence of resistance among isolates obtained from Addis Ababa abattoir was high compared to isolates from other sources particularly to beta lactam antimicrobials and sulfisoxazole (Table 8).

One of the two K. *pneumoniae* isolates from Menilik II Referral Hospital wastewater showed intermediate resistance to ciprofloxacin, streptomycin and cefoxitin. In the wastewater form

Addis Ababa Abattoir, one isolate showed intermediate resistance to ciprofloxacin and streptomy cin. Two *Klebsiella pneumoniae* isolates from Kebena River showed intermediate resistance to ciprofloxacin. From Little Akaki river one isolate was identified as *Klebsiella pneumoniae* which showed intermediate resistance to ciprofloxacin and streptomycin.

Table 8. Antimicrobial susceptibility profile of *Klebsiella pneumoniae* isolated from Menilik II Referral Hospital, Addis Ababa Abattoir, Kebena River, Little Akaki River and Kality WWTP

Drug	No. of isolates exhibiting resistance					No.(% resistant of all isolates) (N=8)
	Menilik II Referral hospital (N=2)	Addis Ababa Abattoir (N=2)	Kebena River (N=2)	Little Akaki River (N=1)	Kality WWTP (N=1)	
amoxicillin/clavulanic acid	1	2	1	0	1	5(62.5)
Ciprofloxacin	0	0	0	0	1	1(12.5)
Gentamicin	0	0	0	0	0	0
Streptomycin	0	0	0	0	0	0
sulfamethoxazole/trimetoprim	2	0	0	0	0	2(12.5)
Trimethoprim	1	0	0	0	1	2(25)
Tetracycline	1	0	1	1	0	3(37.5)
Ampicillin	2	2	2	1	1	8(100)
Cephalotin	2	2	2	1	1	8(100)
Cefoxitin	0	2	1	0	1	4(50)
Ceftriaxone	0	2	0	0	0	2(25)
Sulfisoxazole	1	2	0	0	0	3(37.5)

Kelbsiella oxytoca was not isolated from Addis Ababa Abattoir, Kebena River and Kality WWTP, whereas only one isolate each from two hospital wastewater samples and another from Little Akaki River were isolated. Despite difference in source of isolation, all *K. oxytoca* isolated in this study were resistant to ampicillin, cephalothin and amoxycillin+cauvlanic acid (Table 9). All of the three *K. oxytoca* isolates showed intermediate resistance to ciprofloxacin.

Table 9. Antibiotic sensitivity of *Klebsiella oxytoca* isolated from TASH, Menilik II Referral Hospital and Little Akaki River

Drug	No. of isolates exhibiting resistance			No.(% resistant of all isolates), (N=3)
	TASH (N=1)	Menilik II Referral hospital (N=1)	Little Akaki River (N=1)	
amoxicillin/clavunic acid	1	1	1	3(100)
Ciprofloxacin	0	0	0	0
Gentamycin	0	0	0	0
Streptomycin	0	0	0	0
sulfamethoxazole/trimetoprim	0	0	0	0
Trimethoprim	0	0	0	0
Tetracycline	0	0	0	0
Ampicillin	1	1	1	3(100)
Cephalotin	1	1	1	3(100)
Cefoxitin	0	0	0	0
Ceftriaxone	0	0	0	0
Sulfisoxazole	0	0	0	0

6.6. Antimicrobial susceptibility of *Salmonella* isolates

Although *Salmonella* was isolated from all study sites, recovery of *Salmonella* was more common in samples from downstream Rivers and Addis Ababa Abattoir than the two hospital wastewater samples and rate of resistance to more number of drugs was also observed in isolates obtained from river than those from hospitals. Among 12 *Salmonella* isolates investigated in this study, all (100%) of them were resistant to ampicillin and cephalothin, 7(58.3%) were resistant to tetracycline and cefoxitin 7(58.3%). Interestingly, all isolates were susceptible to ciprofloxacin , gentamicin and ceftriaxone (Table 10). The isolate from Menilik II Referral Hospital wastewater showed intermediate resistance to amoxicillin/clavunic acid. One of the two isolates from Addis Ababa Abattoir showed intermediate resistance to amoxicillin/clavulanic acid and ciprofloxacin. Two of the three isolates showed intermediate resistance to ciprofloxacin, streptomycin and one isolate showed intermediate resistance to gentamycin. One of the two

isolates from Kality WWTP showed intermediate resistance to amoxicillin/clavunic acid and ciprofloxacin. Two of the three isolates from Little Akaki River showed intermediate resistance to amoxicillin/clavunic acid and one showed intermediate resistance to streptomycin.

Table 10. Antimicrobial susceptibility profile of *Salmonella* spp. isolated from TASH, Menilik II Referral Hospital, Addis Ababa Abattoir, Kebena River, Little Akaki River and Kality WWTP

	No. of isolates exhibiting resistance						No.(%) resistant of all isolates), (N=12)
Drug	**TASH (N=1)**	**Menilik II Referral Hospital (N=1)**	**Addis Ababa Abattoir (N=2)**	**Kebena River (N=3)**	**Little Akaki River (N=3)**	**Kality WWTP (N=2)**	
Amoxicillin+clavulanic acid	0	0	0	2	0	1	3(25)
Ciprofloxacin	0	0	0	0	0	0	0
Gentamicin	0	0	0	0	0	0	0
Streptomycin	0	0	0	0	2	1	3(25)
sulfamethoxazole +trimethoprim	0	0	0	1	0	0	1(8.3)
Trimethoprim	0	0	0	1	0	0	1(8.3)
Tetracycline	0	0	0	3	3	1	7(58.3)
Ampicillin	1	1	2	3	3	2	12(100)
Cephalotin	1	1	2	3	3	2	12(100)
Cefoxitin	0	0	1	2	2	2	7(58.3)
Ceftriaxone	0	0	0	0	0	0	0
Sulfisaxazole	0	0	0	0	2	0	2(16.7)

6.7. Antimicrobial resistance pattern of species of Enterobacteriacea e isolated from wastewater samples

In general, diverse phenotypic resistance pattern was observed for majority of the species of Enterobacteriaceae in this study. Overall; the highest rate of multidrug resistance (MDR) was detected in two *Citrobacter* isolates obtained from TASH wastewater samples in which the two isolates were fully resistant to all 12 antimicrobials tested. A single isolate obtained from Addis Ababa abattoir was also resistant to 9 antimicrobials tested. Similarly 2 isolates of Enterobacter colaceae isolated from TASH wastewater samples also exhibited high level of MDR including resistance to ciprofloxacin and third generation cephalosporin. Two *E.coli* isolates from TASH were also MDR to 11 of 12 antimicrobials tested. In general multiplicity of MDR is more commonly observed in *Citrobacter, Enterobacter* and *E. coli* isolates and it is more common in isolates obtained from hospitals particularly TASH compared to other sources. Majority of *Salm onella* isolates in the current study were not MDR compared to other species and the largest number of drugs to which isolates were resistant was observed in an isolate obtained from Kebena River and interestingly unlike other species of Enterobacteriaceae, 3 isolates from the hospitals and Addis Ababa Abattoir house were resistant to only two drugs whereas majority of isolates from downstream water bodies were resistant to more number of drugs (Table 11).

Table 11. Antimicrobial resistance patterns of bacteria isolated from various wastewater samples and tested for susceptibility to 12 antimicrobials and their AMR-index.

Bacterial species(Total No. tested)	Place of collection	R-pattern	No. with this R pattern	Total No. of drugs to which isolate is R	AMR Index
Citrobacter spp. (3)	TASH	Amc,Cpr,Gm,S, Sxt, G, Tmp, TE, Amp,Cf, Fox, Cro,Ctx	2	12	1
	Kality WWTP	Amc,Cpr,G,TE,Amp,Cf,Fox,Cro,	1	9	0.75
Enterobacter aerogenes (3)	Little Akaki river	Amc,TE,Amp,Cf,Fox,Cro, Ctx	1	7	0.58
	AA abattoir	Amc,S,Amp, Cf	1	4	0.33
	AA abattoir	Amc,Amp,Cf, Fox	1	4	0.33
Enterobacter cloacae(6)	TASH	Amc,Cpr,Sxt,G,Tmp,Te,Amp,Cf,Fox,Cro	2	10	0.83
	Menilik II hospital	Amc,Amp,Cf	1	3	0.25
	Kebena river	Amc,Amp,Cf, Fox	1	4	0.33
	Kality WWTP	Amc,Amp,Cf, Fox	1	4	0.33
	Little Akaki river	TE,Amp,Cf	1	3	0.25
Escherichia coli(18)	TASH	Amc, Cpr, S, Sxt, G, Tmp, TE, Amp, Cf, Fox, Cro	2	11	0.91
	TASH	Amc,Tmp, TE, Amp, Cf	1	5	0.41
	TASH	Amc,Cpr,Amp, Cf	1	4	0.33
	Menilik II hospital	Amc, Cpr ,S, TE, Amp, Cf, Fox, Cro	1	8	0.66
	Menilik II hospital	Amc,Cpr,S,Sxt,G ,Tmp,TE,Amp	1	8	0.66
	Menilik II hospital	Amc,Cpr, Amp, Cf	1	4	0.33
	AA abattoir	Amc,Amp, Cf, Fox	1	4	0.33
	AA abattoir	S,Sxt,G,Tmp, TE,AMP, Cf	1	7	0.58
	AA abattoir	Amc,S,Sxt,G,Tmp, TE,Amp,Cf, Fox	1	9	0.75
	AA abattoir	Amc, TE, Amp, Cf, Fox	1	5	0.41
	Kality WWTP	Amc,Amp,Cf, Fox	1	4	0.33
	Kality WWTP	TE,Amp, Cf	1	3	0.25
	Kebena river	TE,Amp, Cf	1	3	0.25
	Little Akaki river	Amc,S,Sxt,G,Tmp, TE,Amp,Cf, Fox	1	9	0.75
	Little Akaki river	Amc,TE,Amp,Cf, Cro	2	5	0.41
Klebsiella oxytoca(3)	TASH	Amc,Amp, Cf	1	3	0.25
	Menilik II hospital	Amc,Amp, Cf	1	3	0.25
	Little Akaki river	Amc,Amp, Cf	1	3	0.25
Klebsiella pneumoniae(8)	Menilik II hospital	Amc,Amp,Cf,S, Sxt,G,Tmp,TE	1	8	0.66
	Menilik II hospital	Amc,Amp,S, Cf	1	4	0.33
	AA abattoir	Amc,Amp, Cf	2	3	0.25
	Kality WWTP	Amc,Cpr,Tmp, Amp,Cf, Fox	1	6	0.5
	Kebena river	Amc,Amp,Cf, Fox	1	4	0.33
	Kebena river	TE,Amp, Cf	1	3	0.25
	Little Akaki river	TE,Amp, Cf	1	3	0.25

Salmonella species(12)	TASH	Amp, Cf	1	2	0.16
	Menilik II hospital	Amp, Cf	1	2	0.16
	AA abattoir	Amp, Cf	1	2	0.16
	AA abattoir	Amp,Cf, Fox	1	3	0.25
	Kality WWTP	Amc,Amp,Cf, Fox	1	4	0.33
	Kality WWTP	Amp,Cf, Fox	1	3	0.25
	Kebena river	Amc,Sxt,Tmp, TE,Amp,Cf,Fox	1	7	0.58
	Kebena river	TE,Amp,Cf	1	3	0.25
	Kebena river	Amc,TE,Amp,Cf, Fox	1	5	0.41
	Little Akaki river	G,TE,Amp,Cf, Fox	1	4	0.33
	Little Akaki river	S,G,TE,Amp,Cf,Fox	1	6	0.5
	Little Akaki river	TE,Amp, Cf	1	3	0.25

Amc= Amoxicillin+clavulanic acid, Cpr= Ciprofloxacin, Gm= Gentamicin, S= Streptomycin , Sxt= sulfamethoxazole+trimethoprim , Tmp= Trimethoprim ,TE= Tetracycline, Amp= Ampicillin, Cf= Cephalotin, Fox= Cefoxitin, Cro= Ceftriaxone, G= Sulfisaxazole

6.8. Extended spectrum beta-lactamase production by resistant isolates

Of the 22 isolates of Enterobacteriaceae tested for production of extended spectrum beta -lactamase using double disc synergy test, only six (27.3%) of the isolates were shown to produce extended spectrum beta-lactamase enzyme. Representative image of double disc synergy test result is shown in Fig. 2. These isolates were one *K. pneumoniae* and one *E. coli* isolated from wastewater sample of Menilik II Referral hospital; one *Citerobacter species*, one *Enterobacter cloaceae* and one *E. coli* isolated from wastewater sample from TASH, one *E. coli* isolate obtained from Little Akaki river. Proportion of isolates producing extended spectrum beta-lactamase among the Enterobactereace isolates examined and relative distribution among different species is shown in Table 12.

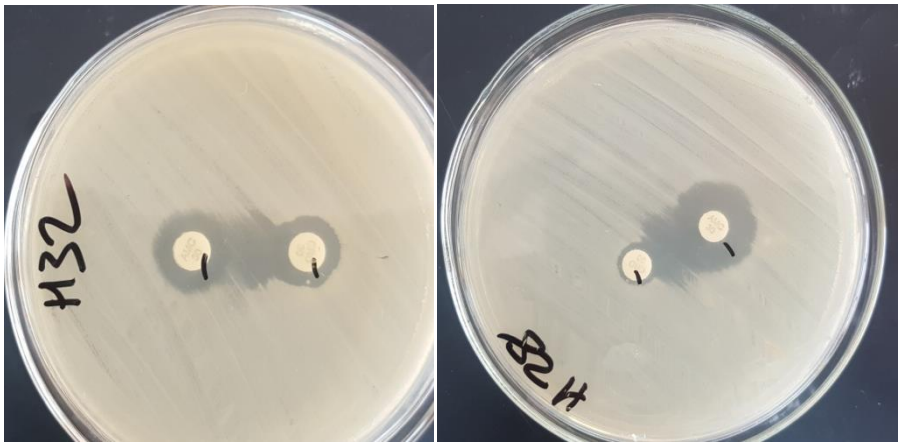

Figure 2. Representative image of result of double disc synergy test; H32 *Citrobacter* species isolated from TASH; H 28; Enterobacter cloaceae from TASH

Table 12. Rate of occurrence of extended spectrum beta-lactamase production among Enterobacteriaceae using double disc synergy test

Bacterial species	DDST result		% positive	Remark
	No. tested	No. positive		
Citrobacter species	3	1	33.3	Positive one was isolated from TASH
E. coli	11	3	27.3	2 from Menilik II Referral Hospital and TASH, while one from Little Akaki river
Enterbacter cloaceae	4	1	25	Positive one from TASH
Klebsiella pneumoniae	3	1	33.3	Positive one from Menilik II Referral Hospital

6.9. Carbapenemase production by multidrug resistant isolates

Modified Carbapenem inactivation assay was conducted for 28 different Enterobacteriaceae which exhibited resistance to ampicillin, cephalothin, cefoxitin and ceftriaxone to examine if the isolates are also capable of producing carbapenemase enzyme. None of these isolates were shown to produce carbapenemase.

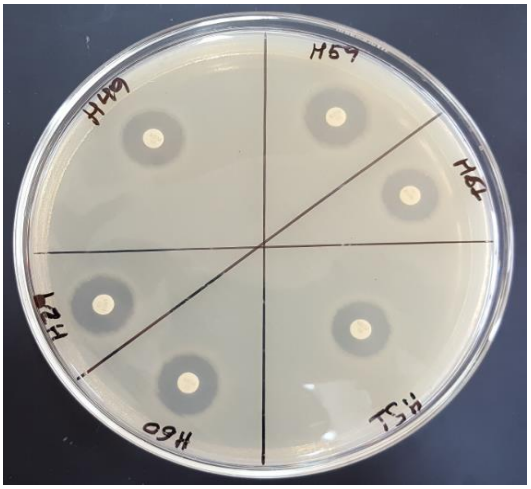

Figure 3. Carbapenemase Production test of Enterobacteriaceae

7. Discussion

In this study, members of the family Enterobacteriaceae (*E. coli, Salmonella* spp, *Enterobacter aerogenes, Klebsiella pneumoniae, Citrobacter spp, Kelbsiella oxytoca* and *Enterobacter cloaca e*) were detected from hospital wastewater, Addis Ababa Abattoir wastewater, Kebena River and Kality WWTP. The detected bacterial species comprised of *E. coli* (32%), *Salmonella* spp. (23%), *Klebsiella pneumoniae* (15%), *Enterobacter aerogenes* (11%), *Citrobacter* spp. (7%), *Kelbsiella oxytoca* (6%) and *Enterobacter cloacae* (6%), respectively. In terms of microbi al diversity, this work tallies with the work of Svantrom (2014), which reported the isolation of *E. coli,* and *Salmonella* in abattoir wastewater effluents. Similarly, in Ado-Ekiti (Nigeria), Oluyege and Famurewa (2015), reported the isolation of *E. coli, Klebsiella* Spp., and *Enterobacter* Spp. from eatery houses at various percentages.

Detection of MDR strains of *Citrobacter* species from TASH wastewater is in line with high rate of antimicrobial use in the hospital and previous reports also showed MDR Eneterobacteriaceae in TASH causing severe morbidity and mortality in hospitalized patients (Seboxa *et al.*, 2015). A high degree of resistance to many antimicrobials including third generation cephalosporins (85.3% and 86.3% for ceftazidime and cefotaxime, respectively), piperacillin (80.5%), and ciprofloxacin (81.9%) was reported in previous study from hospital environment (Mohanty *et al*., 2006). And this is may be due to absence of wastewater treatment plant in the case of TASH. Dissemination of such MDR strains carrying resistance genetic markers may impose high risk of spread of resistance genes. The level of MDR in isolates obtained from Kality WWTP and Little Akaki River was not as high as the one obtained from TASH. However, low level of MDR in strains isolated in this study does not mean that there is no such MDR strain in downstream water bodies since the current isolation procedure is not exhaustive as only single colony was

picked from a plate. Further study will be required to proof the absence of such MDR strain in downstream water bodies.

Detection of *E. coli* was more common in samples from hospitals and abattoir compared to samples from receiving rivers and Kality WWTP. High rate of detection of *E. coli* compared to other species is not surprising as it is one of the commensal organisms commonly available in gastrointestinal tract of humans and animals. Direct and indirect fecal contamination of wastewat er from hospital and Abattoir can easily contaminate the receiving water bodies with potential pathogenic *E. coli* as well as multidrug resistant strains which can horizontally disseminate to other organisms. Although this study did not investigate if the isolated *E. coli* strains in the current study were pathogenic or not, high rate of multidrug resistance was detected in majority of the isolates. Similarly *E.coli* was detected in high concentration from the effluents of hospital wastewater in South Ethiopia (Fekadu *et al.*, 2015).

Among *Enterobacter* species, two *E. cloaceae* isolates were detected in isolates from TASH and they were both MDR to several antimicrobials. *Enterobacter* is one of the common causes of nosocomial infection and also claimed to serve as the reservoir of antimicrobial resistance genes (Regli and Pages, 2015). Both *E. aerogenes* and *E. cloaceae* are causes of important opportunistic nosocomial infections responsible for outbreaks (Pestourie *et al.*, 2014). They are known to acquire numerous mobile genetic elements which contribute to fitness of the organism to colonize several environments and hosts (Pestourie *et al.*, 2014). Horizontal transfer of resistance genes from *Klebsiella pneumoniae* is implicated to be the main reason of wide occurrence of MDR *Enterobacter* species in hospital environment (Regli and Pages, 2015). Therefore, detection of such MDR strains in hospital wastewater samples is an indication of risks associated with potential life-threatening infection as well as possibility of downstream

dissemination of MDR isolates or MDR conferring genetic markers to other bacterial communiti es. A few *Enterobacter* strains isolated from the Rivers and Kality WWTP were not resistant to several drugs unlike those isolates from hospital wastewater. This is presumably due to dilution effect of the environmental sources of *Enterobacter* species which are not exposed to antimicrobials since the current study protocol was not designed to pick MDR isolates selectively. *Enterobacter cloaceae* is ubiquitous in terrestrial and aquatic environments (Mezzatesta *et al.,* 2012). However, as the sample size is not large enough in the current study, we cannot rule out the presence of MDR *Enterobacter* in receiving water bodies.

All of the 18 *E. coli* isolates in this study were resistant to ampicillin and cephalothin. Resistance of *E. coli* to various antimicrobials, particularly to major beta lactam antimicrobials was high in isolates obtained from Hospital wastewater and AA abattoir compared to those obtained from downstream rivers and Kality WWTP. Ciprofloxacin resistance was detected only in isolates obtained from hospital wastewater. This finding of high rate of resistance in hospital wastewater and abattoir, is undoubtedly due to high selection pressure for resistant strains in the hospital and abattoir facilities due to overuse of antimicrobials in these facilities. High rate of resistance of *E.coli* strains to beta lactams from clinical and environmental origin has been reported worldwide (Ash *et al.*, 2002; Jain and Mondal, 2007). Since major mechanism of resistance to beta lactam antimicrobials is due to production of beta-lactamase enzymes encoded by genes carried on various plasmids, these *E. coli* isolates can contribute to horizontal transmission of resistance genes to other bacterial species in the wastewater and receiving downstream water bodies (Alekshu and Levy, 2007).

Unlike other species of Enterobacteriaceae described above, overall resistance rate to *Klebsiella* species was relatively low and no *Klebsiella* isolate was detected from TASH wastewater

sample. Overall occurrence of resistance among isolates obtained from AA abattoir was high compared to isolates from other sources particularly to beta lactam antimicrobials and Sulfisoxazole. *Klebsiella pneumoniae* from Hospital environment is widely known to carry genes coding for resistance to several drugs including those producing extended spectrum beta-lactamase (ESBL) and *Klebsiella pneumoniae* carbapenemase (KPC) production (Paterson *et al.*, 2005; Picao *et al*., 2013).

Salmonella spp were isolated from all wastewater samples; however, detection rate was higher in samples from rivers compared to the hospital and abattoir samples. This is probably due to contamination of rivers by fecal materials of food animals or humans in addition to contamination with hospital and abattoir wastewater. *Salmonella* usually occurs in gastrointestinal tract or feces of various animals like cattle, poultry, small ruminants and pet animals like dogs (Kiflu *et al.*, 2017; Eguale *et al.*, 2016) which can easily access the rivers through direct discharge from the farms to the rivers or through flood during rainy season. High rate of resistance to ampicillin, cephalothin (100%) each and tetracycline (58.3%) is in line with findings for *Salmonella* isolates from food animals and humans in Addis Ababa (Eguale *et al*., 2015; Eguale *et al.*, 2016). Vegetables sold in Addis Ababa market were shown to be contaminated by *Salmonella* in previous study (Guchi and Ashenafi, 2010). Resistance to tetracycline is particularly high presumably due to widespread use of oxytetracycline in livestock production in the country (Beyene *et al.*, 2016). Contrary to reports of MDR strains of non-typhoidal *Salmonella* causing invasive disease in young children from TASH (Beyene *et al.*, 2011), a single *Salmonella* isolate resistant only to ampicillin and cephalothin was detected in the current study

from wastewater samples from TASH. However, this may not represent the real situation of the hospital environment as sampling was done only twice in this study.

In general, high level of MDR as high as resistance to all 12 tested drugs in *Citrobacter* species from TASH wastewater, and 11 drugs in *E. coli* isolated from wastewater sample from the same hospital were recorded in this study. Similarly, high level of MDR to as high as to 8 antimicrobials in *E. coli* from Menilik II Referral Hospital wastewater and 9 antimicrobials in *E. coli* from Addis Ababa abattoir were recorded. This shows that wastewater from these sites particularly TASH is serving as a major hot spot for MDR isolates and drug resistance genes. Interestingly a single *Citrobacter* species isolated from Kality WWTP was also resistant to 9 antimicrobials including ciprofloxacin and ceftriaxone. This isolate probably originated from TASH or other health facility in the way towards treatment plant. The highest AMR index of 1 was recorded in *Citrobacter* isolate from TASH wastewater followed by 0.91 for *E. coli* from the same source. *E. coli* and *Citrobacter* are known to be the major causes of nosocomial infection and reservoirs of acquired resistance genes in the hospital environment (Seboxa *et al.,* 2015). The lowest AMR index of 0.16 was recorded for *Salmonella* species from TASH, Menilik II Referral Hospital and Addis Ababa abattoir all of them sharing same resistance pattern ampicillin and cephalothin. Interestingly, unlike other species, *Salmonella* isolates from rivers were resistant to more number of drugs compared to those from Hospitals and Addis Ababa abattoir in the current study. This is probably due to contamination of rivers with other sources or due to acquisition of resistance genes from other bacteria in the river. Previous study in Addis Ababa showed that some dairy farms in Addis Ababa are contaminated with MDR *Salmonella* and the drainage from these farms may access the rivers (Addis *et al.,* 2011; Eguale *et al.,* 2016).

In this study, 22 isolates of species belonging to family of Enterobacteriaceae resistant or intermediately resistant to 2nd and 3rdgeneration cephalosporin were tested for production of extended spectrum beta-lactamase (ESBL) using double disc synergy test of which only six (27.3%) of the isolates were shown to be ESBL producers. Majority of ESBL producers were derived from hospitals wastewater. This could be due to the fact that selection for ESBL producing organisms could easily occur in hospital environment due to overuse of multiple drugs in patients admitted to hospital and suffering from various nosocomial infections. The high frequency of ESBLs producing Enterobcteriaceae is reported to be due to the selective pressure created by the frequent use and overuse of the 3rd generation cephalosporins (Dhara *et al.*, 2012). The observed MDR phenotype to beta lactams including third generation cephlosporins in the remaining isolates which are negative for ESBL production could be due to production of broad spectrum beta-lactamases, inhibitor-resistant beta-lactamases and Cephalosporinase over production (Drieux *et al.,* 2008).

In this study, carbapenemase production was not demonstrated in any of the isolates which is contrary to the report in other countries where crbapenemase producing Enterobacteriaceae has been reported in hospital wastewater (White *et al.,* 2016). The low usage rate of carbapenem antimicrobials in hospitals in Ethiopia may be the reason for absence of carbpenemase production in the current isolates. Overuse of carbapenems may promote the recovery selection for carbapenem resistant Enterobacteriaceae (CPE). As recent report from children suspected of septicemia and urinary tract infections in Addis Ababa showed production of CPE in 12.1% of the isolates (Legesse *et al.,* 2017), absence of carbapenemase production in the current isolates doesn't rule out presence of CPE strains in wastewater samples in the current study area at least for those from Hospital.

8. Conclusion and recommendations

This study demonstrated abundance of antimicrobial resistant Enterobacteriaceae in wastewater from Hospitals and Addis Ababa Abattoir as well as receiving downstream water bodies. High level of antimicrobial resistance was particularly detected in *Citrobacter* and *E. coli* isolates from Hospital waste water. The use of downstream rivers contaminated with this MDR bacterial pathogens cause's major public health risks for people using these rivers for irrigation of vegetable farms along Kebena River as well as Little Akaki river residents of Addis Ababa who use the vegetables grown by these farmers. These MDR bacterial species may also transmit their resistance genes to other pathogenic or commensal organisms in the water bodies and also in the gut of humans and animals further increasing spread of antimicrobial resistance.

Based on the above conclusive remarks the following recommendations are made:

- Hospitals and Abattoir wastewater should be treated by appropriate wastewater treatment plants before being released into the environment.
- Antimicrobial susceptibility profile of Enterobacteriaceae in hospital effluent should be regularly monitored to reduce dissemination of resistant bacteria into the environment
- Further studies should be conducted in the region to reveal healthcare liquid waste management and microbiological quality of effluents discharged into receiving environment.

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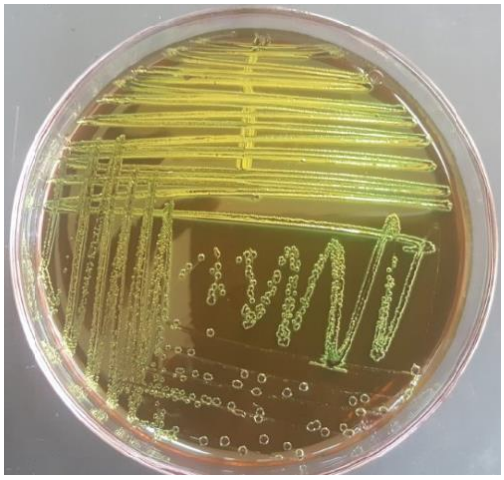
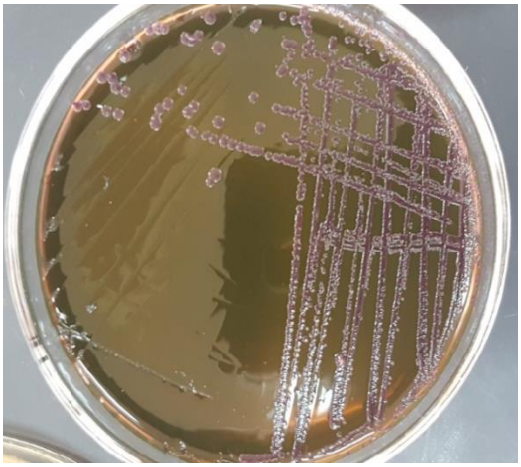
10. Appendixes

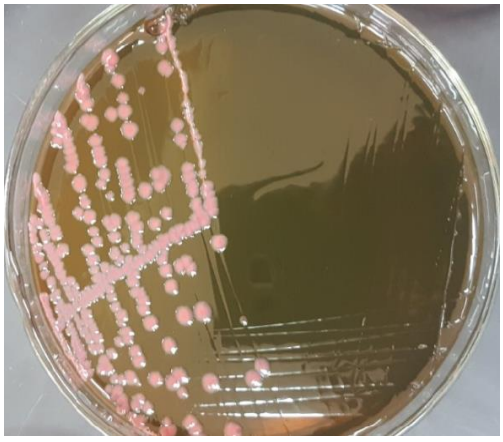
Appendix 1 Isolation of *Klebsiellas pp*, *Escherichia coli*, *Enterobacter spp* and *Citrobacter spp* with selective culture media Eosin methylene blue (EMB)



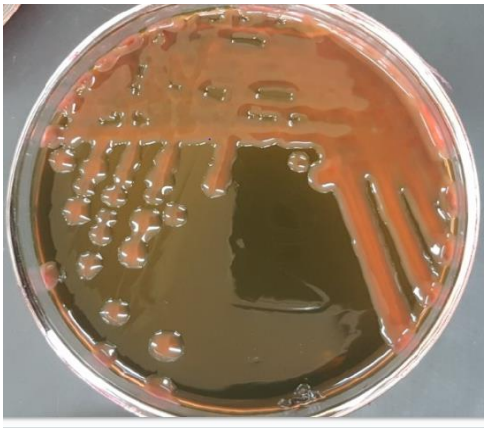
Escherichia coli



Enterobacter spp

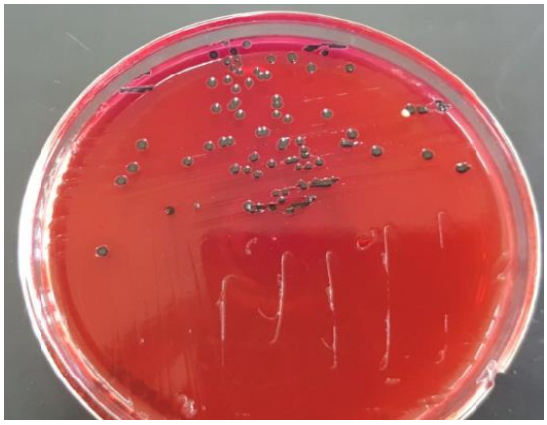


Citrobacter spp.



Klebsiellas pp

Appendix 2 Isolation of *Salmonellas pp*. with selective culture media Xylose Lysine tergitol 4 (XLT-4)

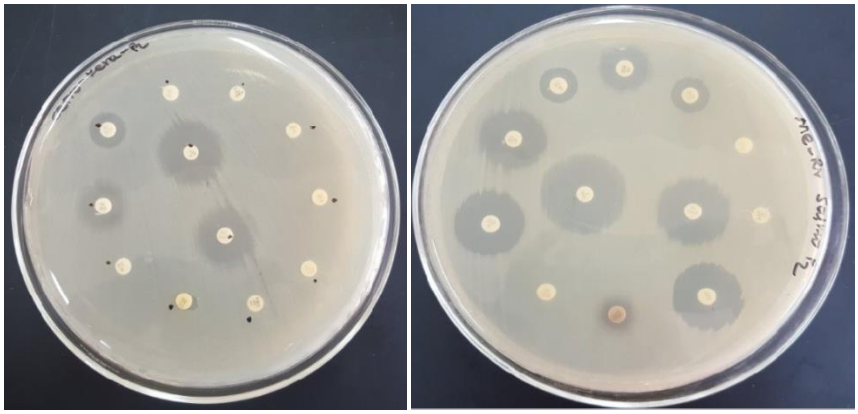


Salmonella

Appendix 3 Identification result of Enterobacteriaceae using biochemical tests

Appendix 4 Identification of Enterobacteriaceae using API 20E kit result

Appendix 5 Resistance pattern of Enterobacteriaceae to selected antimicrobials



Appendix 6 Little Akaki River irrigated farm land

Appendix 7 Kebena River (A) and farm land irrigated with Kebena River (B)

A B

Appendix 8 Addis Ababa Abattoir final effluent

Declaration

I, the under signed, declare that this thesis is my original work. It has never been submitted in any institution and that all sources of materials used for thesis have been acknowledged.

Name: Hemen Tesfaye

Signature _____

Date _____

This thesis has been submitted for examination with our approval as

Advisors: 1. Adey Feleke (PhD) Signature _____

2. Tadesse Eguale (PhD) Signature _____

Co Advisor: 3. Ato Haile Alemayehu Signature _____