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ASSESSMENT OF ANTIMICROBIAL RESISTANCE OF BACTERIA ISOLATED FROM HOSPITAL AND NON-HOSPITAL SEWARAGE SYSTEMS IN ADDIS ABABA, ETHIOPIA

By

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ASSESSMENT OF ANTIMICROBIAL RESISTANCE OF BACTERIA ISOLATED FROM HOSPITAL AND NON-HOSPITAL SEWARAGE SYSTEM IN ADDIS ABABA, ETHIOPIA

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Acronyms

AAU: Addis Ababa University

AMR: Antimicrobial Resistance

ARB: Antibiotic Resistant Bacteria

ARGs: Antibiotic Resistant Genes

APHA: American Public Health Association

CLSI: Clinical Laboratory Standards Institution

DMIP: Department of Microbiology, Immunology and Parasitology

EPA: Environmental Protection Agency

EPHI: Ethiopian Public Health Institute

FMOH: Federal Minister of Health

HGT: Horizontal Gene Transfer

HWW: Hospital Wastewater

MDR: Multi Drug Resistance

MRSA: Methicillin Resistant *staphylococcus aureus*

MSSA: Methicillin Susceptible *Staphylococcus aureus*

NCCLS: National Committee for Clinical Laboratory Standards

STPs: Sewage Treatment Plants

USA: United States of America

VRE: Vancomycin Resistant *Enterococci*

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Abstract

Background: -Large quantities of antimicrobials are used in hospitals for patient care. Antibiotics are partially metabolized and residual quantities reach hospital sewage, exposing bacteria to a wide range of biocides that could act as selective pressure for the development of resistance.

Objectives: The aim of this study was to isolate selected common bacterial pathogens and assess antimicrobial resistance of bacteria isolated from sewage released from hospitals and non-hospital sewerage system in Addis Ababa, Ethiopia

Methods: A cross-sectional study was conducted from August 2016-December 2017 in hospital and non-hospital sewage. A total of 220 hospital and non-hospital sewage samples were collected twice a week for five weeks for bacteriological analysis and susceptibility testing. Pathogenic and potentially pathogenic bacteria were isolated on selective bacteriologic media and antibiotic susceptibility tests were performed using Kirby-Bauer disk diffusion method. All methods were used according to standard methods for examination of water and wastewater.

Results: A total of 220 waste water samples were processed for the presence of drug resistant pathogens. From these total samples 506 bacterial isolates were isolated and of these 327 (64.6%) were from hospital environment and 179 (35.4%) were from non-hospital environment. The most frequently identified bacterium was *Pseudomonas spp.* 160(31.6%) followed by *E.coli* 108(21.34%); *Klebsiella spp*.76 (15%); *Citrobacter spp.* 50 (10%); *Staphylococcus aureus* 37 (7.3%);*Enterobacter* 14(2.8%); and 57(11.26%) were other gram positive and gram negative bacteria. Hospital and non-hospital isolates were subjected to antimicrobial susceptibility testing. The percent of resistance for Gram-negative bacteria to 9 antibiotics were as follows: Ampicillin (100% and 95.5%), Imipenem (0% and 0%), Ceftriaxone (75% and 26.9%), Ceftazidim (91.9% and 65.7%), Gentamicin (17.7% and 4.5%), Ciprofloxacin (36.2% and 8.9%), Kanamycin (27.3% and 4.5%), Chloramphenicol (19.6% and 6%) and Cefotaxime (96.5% and 89.9%)for hospital and non-hospital wastewater, respectively. Likewise, the rate of resistance for *S.aureus* against tested drugs was: Ampicillin (100% & 100%), Amoxicillin (100% & 83%) Chloramphenicol (28% & 17%), Ciprofloxacin (16% & 8%), Ceftriaxone (20% & 8%), Gentamicin (12% & 0%), Cefotaxim (40% & 25%), and Erythromycin (20% & 0%) for bacteria isolated from hospital and non-hospital wastewater, respectively.

Conclusion: This study showed that both hospital and non-hospital environments harbor similar types of bacteria, but the hospital environment contains significantly higher number and antibiotic resistance rates of each bacteria types. The contamination of hospital sewage more than that of the non-hospital one by antibiotics or other pollutants lead to the rise and dissemination of multidrug resistance due to selection pressure.

Key words: Hospital environment, Antimicrobial resistance, Non-hospital environment, Wastewater

1. Introduction

Sewage from hospitals , usually referred to as hospital waste, is defined as a special category of waste which comprises of all wastes, biological or non-biological that is discarded from hospitals/health care centers and not intended for further use (Oyeleke and Istifanus, 2008). The important consumption of water in hospitals gives significant volumes of waste water loaded with microorganisms (the majority of which being pathogenic), heavy metals, toxic chemicals and radioactive elements (Kummerer, 2004)

Hazardous medical waste consists primarily of chemicals and discarded cytotoxic drugs which find their way into the environment due to improper usage and indiscriminate disposal. Their presence in the environment have been reported to pose serious environmental health risk due to their toxic, genotoxic and/or carcinogenic effect (Oyeleke and Istifanus, 2008; Akter and Kazi,1999) and could have potential negative effects on the biological balance of natural environment.

Among the health risks associated with wastes of health facility origin, the prominent and most serious one is their potential to contain antimicrobial resistant pathogens. Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. Hospitals provide an environment conducive to multi drug resistant bacteria, making treatment options limited and expensive (Magiorakos *et al*, 2011).Sewerage systems also carry other waste materials from the community and industry and so MDR bacteria must survive a long, hostile transition route, including final disinfection, before they are released into surface waters (Katouli, 2012).In spite of the huge potential for negative health impacts, however, not enough is known about their release and survival from hospital wastewater, through the sewerage system and finally into treated effluent released by sewage treatment plants (STPs) into the environment.

The public health impact of the release of drug resistant bacteria to the receiving environment involves a number of points. First, if the resistant bacteria are carrying a transmissible gene, they transfer resistant genes to other community of pathogenic bacteria so that infection caused by the latter are usually difficult to treat, and it also decreases the antibiotic pool for the treatment of other bacterial infections. Second, these organisms may act as vectors or reservoirs of resistant genes because antibiotics exert selection in favor of resistant bacteria by

killing or inhibiting growth of susceptible bacteria; resistant bacteria can adapt to environmental conditions and serve as vectors for the spread of antibiotic resistance (Kruse *et al*, 1999).Third, there will be increased nosocomial infection. Fourth, if infection occurs, it will increase the costs of treatment and hospitalization (Fekadu *et al,* 2015).

The main risk for public health is that resistance genes are transferred from environmental bacteria to human pathogens (Kruse *et al*, 1999; Wegner *et al*, 1999). The large volume of antibiotics used in hospitals and private households which are released into effluent and municipal sewage indicates a selection pressure on bacteria (Kummerer and Henniger, 2003).Because waste effluent from hospitals contains high numbers of resistant bacteria and antibiotic residues at concentrations able to inhibit the growth of susceptible bacteria (Grabow and Prozesay, 1973; Linton *et al*, 1974). As a result, hospital waste effluent could increase the numbers of resistant bacteria in the recipient sewers by both mechanisms of introduction and selection for resistant bacteria (Al-Ahmed *et al*, 1999)

Resistant infections increase the time patients stay in hospitals and patient mortality. For example, research found a 5.02 fold increase in mortality relative risk with a cephalosporin resistant *Enterobacter* infection, a 20.1% vs. 6.7% surgical wound site mortality due to Methicillin resistant vs. Methicillin susceptible *S. aureus*, respectively,and a3-fold increase in mortality and 1.7-fold increase in hospital stay due to multi-drug resistant *Pseudomonas aeruginosa* (Corgrove, 2006)

Mortality from a resistant infection increases for multiple reasons, but, primarily, because of a delay in effective treatment of the resistant infection and increased need for surgery, and other procedures (Corgrove, 2006). Comparison of onset of adequate treatment for susceptible versus resistant strains illustrates how extensively delayed treatment affects patient outcomes. The median interval between obtaining a sample for culture and initiating antibiotics is 51 hours for resistant infections vs. 16 hours for susceptible infections (Corgrove, 2006; Lautenbatch *et al,* 2005). The numbers of patients who receive effective treatment within 24 hours are 36% vs. 68% for resistant and susceptible strains, respectively (Corgrove, 2006; Lautenbatch *et al,* 2005). The numbers of patients who receive effective treatment within 48 hours are 48% vs. 90% for resistant and susceptible strains, respectively (Corgrove, 2006; Lautenbatch *et al,* 2005). These

numbers show that within two days, less than half of the people with resistant infections receive the proper treatment.

1.1. Statement of the problem

Despite the advance in antimicrobial therapy worldwide, the prevalence of infectious disease caused by antimicrobial resistant organisms is increasing . No region in the world has been excluded from the inexorable spread of increasingly drug-resistant bacteria. Hence, antimicrobial resistance (AMR) is now a serious global phenomenon. It occurs, among others, due to releasing residual quantities of antimicrobials in hospital sewerage system that reaches wastewater where hospital microflora are exposed to it. Study conducted in India showed that presence of different antibiotics in hospital effluent and resistance was parallel to antibiotic concentration (Diwan *et al*., 2009) . Similarly, many researches conducted in different countries have shown heavy presence of antibiotic resistant bacteria in hospital effluents (Iversen *et al*, 2002). For instance, study conducted in Nigeria showed that resistance to clinically relevant antibiotics among the organisms reported was an indication of the risks posed by the untreated effluents to public health (Adelow *et al*, 2008). It also adds to the increasing evidence about the role of hospital wastewaters as environmental reservoir for multi-drug-resistant bacteria. Another study conducted in Taiwan to compare resistance pattern among clinical isolates and sewage isolates in the same hospital showed significant difference in resistance to Ampicillin (85.6% vs.94.1%), Ampicillin/Sulbactam (31.7% vs. 55.4%), Cefazolin (29.2% vs. 71.5%) and Cefuroxime (20.7% vs. 61.9%) between clinical and sewage coliform isolates, respectively, showing how important hospital sewage is for spread of antibiotic resistant isolates (Yang *et al*., 2009)

The resistant bacteria evolve and are selected by long-term environmental exposure to the low concentrations of antibiotics (Aparecida *et al*, 2000). In this regard, as most researches indicated, Hospital effluents tested contain different types of antimicrobial drug resistant pathogenic and potentially pathogenic bacteria which are released into receiving water bodies. For example study conducted in Bangladesh showed that 59 *E. coli*, 29 *KlebsiellaPneumoniae subsp. Pneumoniae*, 3 *Klebsiellapneumoniae subsp. Ozaenae* and 5 *Salmonella spp*. isolates were recovered from hospital waste water samples and most of the

isolates werenew variants resistant to almost 20 antibiotics used in this study(Rabbini *et al*, 2012).

One of the impacts of high prevalence of drug resistant pathogens is the economic burden on nations. The economic burden continues to rise as the number of resistant infections as well as the number of drugs to which each microorganism is resistant increases. For examples methicillin resistant *S. aureus* is one of the pathogens mostly isolated from hospital waste water. For instance, while median total cost for MSSA primary nosocomial infections in the USA is only $9,661,the median cost for MRSA primary nosocomial infections is $27,083, showing approximately 3-fold increase in hospital costs from resistance (Murray *et al*, 1999)

Similarly, study conducted to compare prevalence of multidrug resistance of bacteria isolated from hospital waste water and non-hospital waste in North West Ethiopia showed that among the waste water samples tested, diverse drug resistant bacterial isolates were recovered and of these 65 (57.5%) were from hospital environment and 48 (42.5%) were from non-hospital environment(Moges *et al*, 2014).In this study, the most frequently identified bacterium was *Klebsiella spp*.30 (26.6%) followed by *Pseudomonas spp*. 19(16.8%), *E. coli* (11 .5%) and *Citrobacter spp.* (11.5%), and *Staphylococcus aureus* (8.2%). The overall prevalence of multiple drug resistance (MDR) in this study was 79/113 (69.9%).Moreover, MDR in hospital environment was found to be 53/68 (81.5%) while in non-hospital environment it was 26/48(54.2%). (Moges *et al*., 2014).

A study carried out in South Ethiopia that assessed disinfectant and antibiotic resistant bacteria in Hospital waste water at Yirgalem Hospital(YAH) and Hawassa University referral Hospital(HURH) showed that pathogenic (*Salmonella, Shigella* and *S. aureus*) and potentially pathogenic (*E. coli*) bacteria were detected from effluents of both hospitals. Salmonella isolates from YAH effluent were resistant to Ceftriaxone, Tetracycline and Doxycycline, whereas from HURH effluent the isolates were resistant to Gentamycin in addition to the above three antibiotics. Similarly, while *S. aureus* from YAH effluent was resistant to Penicillin, Ampicillin and Amoxicillin, isolates from HURH were resistant to Gentamycinin addition to the above three antibiotics (Fekadu *et al*, 2015)

What is bothering in this regard is that in Ethiopia most hospitals do not have wastewater treatment plant, which worsens the problem because the untreated wastewater are released to rivers, streams or disposed to underground, where some of the water is used for drinking, domestic use, irrigation and recreational purpose. Particularly, rivers are one of the major sources of water directly or indirectly for human and animal consumption, its pollution may contribute to the maintenance and even the spread of bacterial antibiotic resistance (Masdaghinia *et al*, 2009).Thus, understanding the prevalence of antibiotic resistant microbial pathogens from wastewater should be of public health interest since it shades light on the magnitude of the problem, which is vital to devise appropriate intervention strategies. Therefore, the research project presented here was conducted to produce important information that would contribute towards this goal by way of inputting relevant data.

1.2. Significance of the study

Antimicrobial resistance is driving up health care costs, hospital stay, increasing the severity of disease, and increasing the death rates from certain infections. Other than indiscriminate use of antibiotics in human medicine, animal husbandry and agriculture, inappropriate health facility waste disposal may disrupt the microbial balance in favor of resistant bacteria. These conditions become major public health problem especially in developing country including Ethiopia. To limit its public health impact, critical evaluation, treatment and periodic assessment of effluents released to receiving environment is mandatory. Currently, investigation into resistance pattern to common antibiotics in Hospital effluents is scarce in Ethiopia. Therefore this study aims at assessing the resistance pattern of bacteria isolated from hospital and non-hospital effluents. The findings obtained from this study is hoped to provide data for policy makers, health care workers and local authorities so that these responsible bodies would recognize the need for upgrading Hospital effluent management system, and proper antibiotic usage. Thus, this may assist in future planning in an attempt to reduce antibiotic resistance and associated burdens. In addition, the resulting generated data will serve as base-line for further future similar but larger studies.

2. Literature review

Antimicrobial resistance in both pathogenic and communal bacteria is increasing steadily. Failure of antibiotic resistant bacteria containment is responsible for this expansion. Healthcare effluent acts as the store house of harmful infectious pathogens. Potential health risk includes spreading of diseases by these pathogens and wide dissemination of antimicrobial resistance genes, because large quantities of antibiotics are used in hospitals for patient treatment. Most of the antibiotic taken by the patients are partially metabolized and excreted through feces and urine.

2.1. MECHANISMS OF ANTIMICROBIAL RESISTANCES

Prior to the 1990s, the problem of antimicrobial resistance was never taken to be such a threat to the management of infectious diseases. But gradually treatment failures were increasingly being seen in health care settings against first-line drugs and second-line drugs or more. Microorganisms were increasingly becoming resistant to ensure their survival against the arsenal of antimicrobial agents to which they were being bombarded. They achieved this through different means but primarily based on the chemical structure of the antimicrobial agent and the mechanisms through which the agents acted. The resistance mechanisms therefore depend on which specific pathways are inhibited by the drugs and the alternative ways available for those pathways that the organisms can modify to get a way around in order to survive (WHO, 2014 and Cohen *et al*, 2000). Resistance can be described in two ways:

a) **Intrinsic or natural or passive** whereby microorganisms naturally do not possess target sites for the drugs and therefore the drug does not affect them or they naturally have low permeability to those agents because of the differences in the chemical nature of the drug and the microbial membrane structures especially for those that require entry into the microbial cell in order to affect their action. An example of natural resistance is *Pseudomonas aeruginosa*, whose low membrane permeability is likely to be a main reason for its innate resistance to many antimicrobials. Other examples are the presence of genes affording resistance to self-produced antibiotics, the outer membrane of Gram-negative bacteria, absence of an uptake transport system for the antimicrobial or general absence of the target or reaction hit by the antimicrobial (Wish, 1999 ; Toma and Deyno, 2015).

b) **Acquired or active resistance**, the major mechanism of antimicrobial resistance, is the result of a specific evolutionary pressure to develop a counterattack mechanism against an antimicrobial or class of antimicrobials so that bacterial populations previously sensitive to antimicrobials become resistant. This type of resistance results from changes in the bacterial genome. Resistance in bacteria may be acquired by a mutation and passed vertically by selection to daughter cells. More commonly, resistance is acquired by horizontal transfer of resistance genes between strains and species. Exchange of genes is possible by transformation, transduction or conjugation (figure 1) (Yoneyama, *et al*. 2006 and Langton *et al*. 2005). Acquired resistance mechanisms can occur through various ways.

Mechanisms for acquired resistance:- the presence of an enzyme that inactivates the antimicrobial agent, the presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent, a mutation in the antimicrobial agent's target(which reduces the binding of the antimicrobial agent), post-transcriptional or post-translational modification of the antimicrobial agent's target(which reduces binding of the antimicrobial agent), reduced uptake of the antimicrobial agent , active efflux of the antimicrobial agent, over production of the target of the antimicrobial agent, expression or suppression of a gene in vivo in contrast to the situation in vitro and previously unrecognized mechanisms (Langton, *et al.* 2005; Toma and Deyno, 2015)

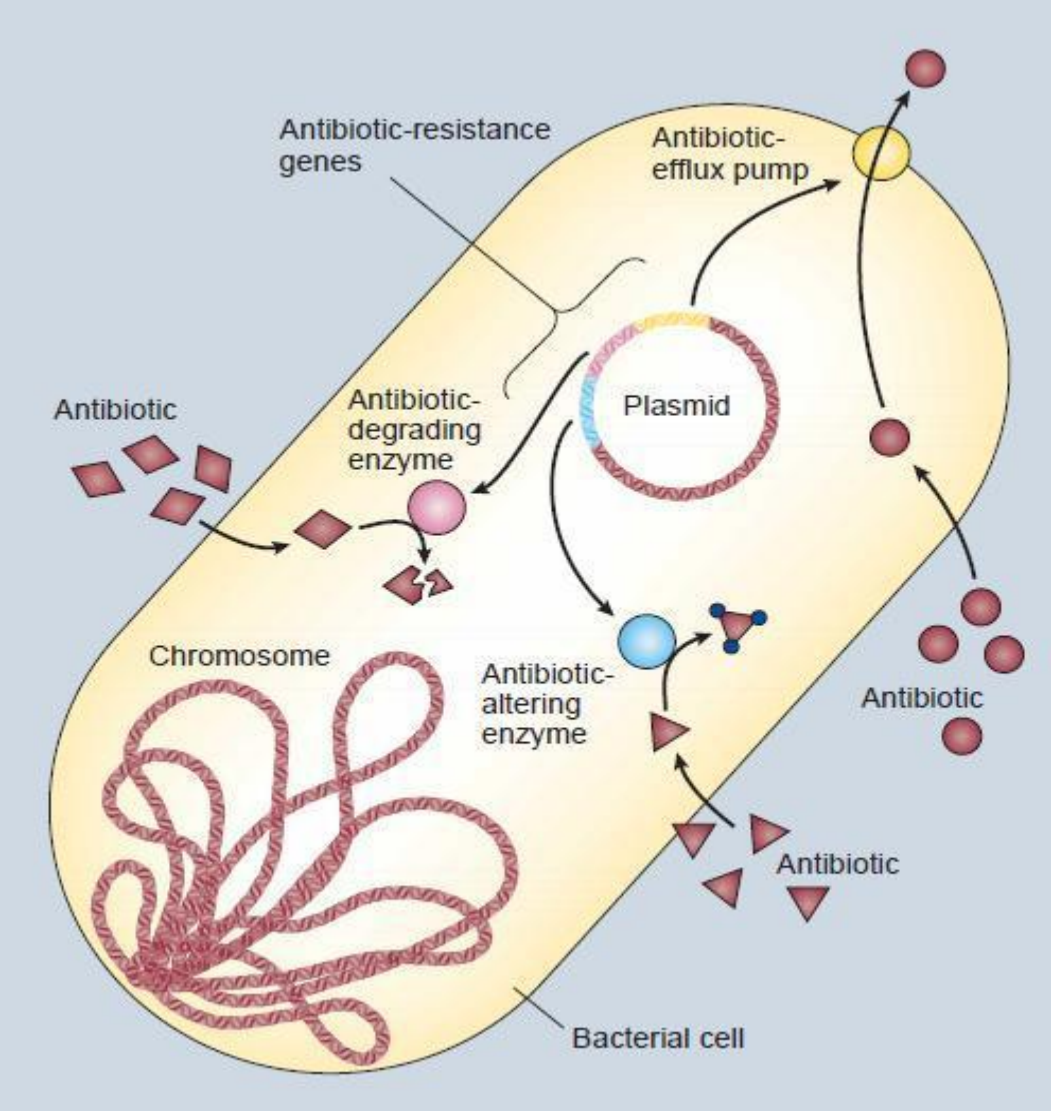


Fig 1: Biological mechanisms of antibiotic resistance in bacteria (Rabbani, *et al*. 2012).

2.1.1. Dessimination of Antimicrobial Resistance

The intercellular spread of the genetic determinants of resistance to antimicrobial agents is facilitated by mobile genetic elements, such as conjugative plasmids and conjugative transposons. The antibiotic resistance genes in these elements are often located within transposons and/or integrons, elements that facilitate the intracellular movement of genes (Rabbani *et al*. 2012).

Under the selection pressure from different antibiotics in the environment, exposed bacterial population may evolve ‗super bugs‗ capable of resisting a wide spectrum of antibiotics through multiple gene transfer and exchange process (Fig 2) (Rabbani *et al*., 2012).Drug resistance genes can be spread from one bacterium to another through various mechanisms such as plasmids, bacteriophages, naked DNA or transposons (tn). Chromosomal genes can be also transferred: they are acquired by one bacterium through the uptake of naked DNA released from another microorganism. This transfer process, called transformation.

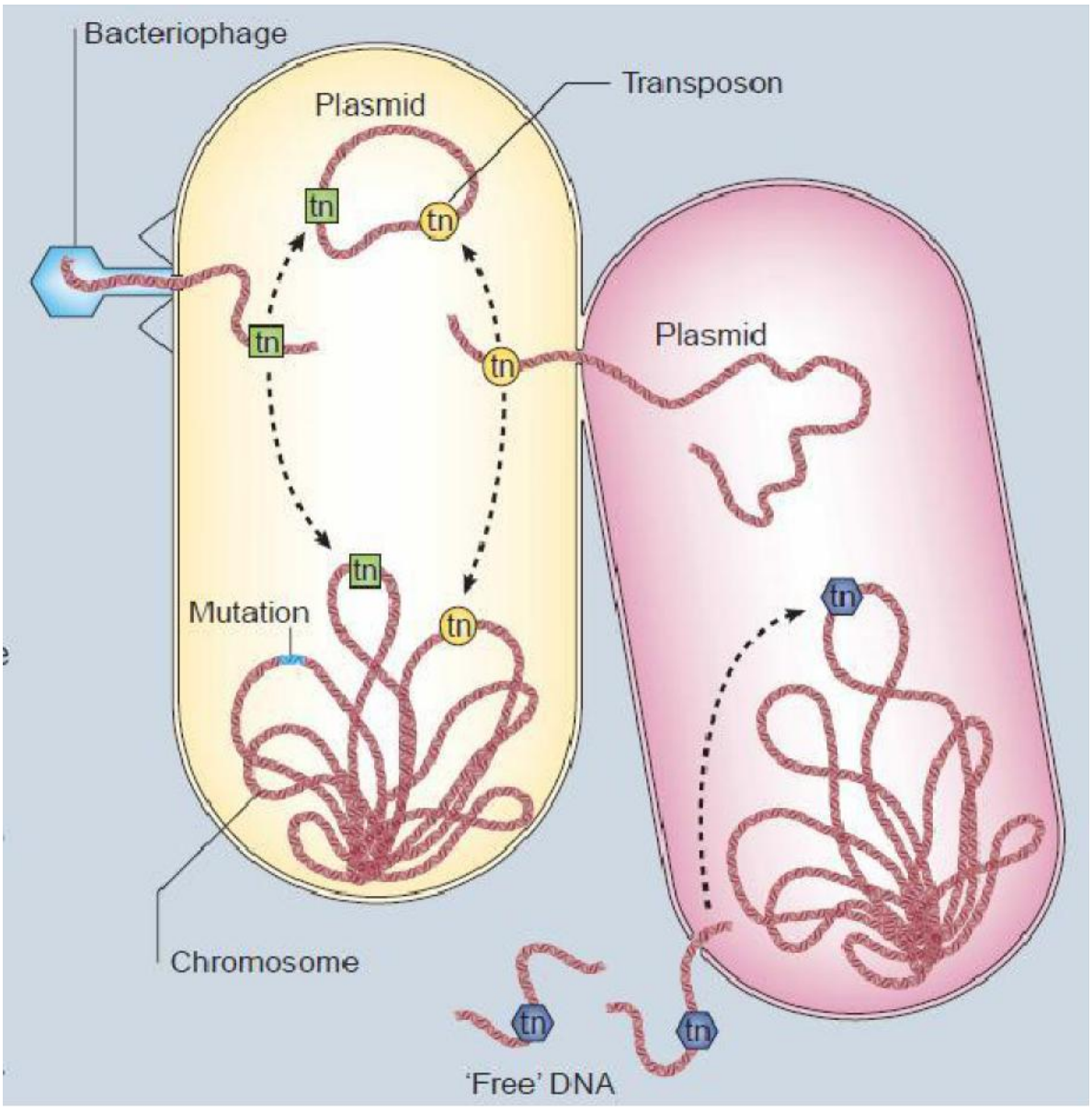


Fig 2.The genetics and spread of drug resistance (Rabbani *et al*, 2012).

2.1.2. Common Bacteria resistant to antibiotics

Some bacteria have developed resistance to antibiotics that were once commonly used to treat them. For example, *Staphylococcus aureus* ('golden staph' or MRSA) and *Neisseria gonorrhoeae* (the cause of gonorrhoeae) are now almost always resistant to benzyl penicillin. In the past, these infections were usually controlled by penicillin(Bartlett, *et al*. 2013).

The most serious concern with antibiotic resistance is that some bacteria have become resistant to almost all of the easily available antibiotics. These bacteria are able to cause serious disease and this is a major public health problem. Important examples are: methicillin-resistant *Staphylococcus aureus (MRSA),* vancomycin-resistant *Enterococcus (VRE),* multi-drug-resistant *Mycobacterium tuberculosis (MDR-TB),*carbapenem-resistant *Enterobacteriaceae*(CRE) gut bacteria, *Pseudomonas aerogenosa*, *Acinetobacter*(Bartlett, *et al*. 2013).

2.2.Waste water and waste water treatment

Waste water is liquid waste, which often contains some contaminants that result from the mixing from different sources, discharged by domestic residences, commercial properties, industry, agriculture, pharmaceuticals and hospitals (Zhang *et al,* 2009). Based on its origin, waste water can be classified as sanitary, commercial, industrial, agricultural or surface runoff (Zhang *et al,*2009). Sewage is subset of wastewater that is contaminated with feces or urine. Naturally occurring antibiotic resistant bacteria (ARB) and antibiotic resistant genes(ARGs) in the aquatic environment are selected for and enriched by antibiotics found in sewage and agricultural runoff, which result from the widespread and increased use of antibiotics (Zhang *et al,*2009; Kummerer, 2004). The occurrence and spread of ARB are pressing public health problems worldwide, and aquatic ecosystems are a recognized reservoir for ARB and ARGs (Baquero and Martinez, 2008; Zhang *et al,*2009).

Hospitals are an essential asset of any society, and waste production is inevitable outcome of service delivery. In hospitals water is consumed by various hospital units such as hospitalization, surgery rooms, laboratories, administrative units, laundry, health services, and kitchen; and in the process its physical, chemical and biological quality decreased and converted to wastewater (Amenu, 2014). Health care waste consists of solid, liquid and gaseous waste contaminated with organic and inorganic substances, including pathogenic microorganisms, radiological chemicals,

and partially metabolized antibiotics, which are usually generated from laboratory analysis of tissues and body fluids as well as excreted from patients (Amenu, 2014).

Sewage is the wastewater released by residences, businesses, health care facilities and industries in a community. It is 99.94 percent water, with only 0.06 percent of the wastewater dissolved and suspended solid material (Environmental Protection Agency, 1997).The cloudiness of sewage is caused by suspended particles which in untreated sewage ranges from 100 to 350 mg/l(Environmental Protection Agency, 1997). Pathogens or disease-causing organisms are present in sewage. Coliform bacteria are used as an indicator of disease causing organisms. Sewage also contains nutrients (such as ammonia and phosphorus), minerals, and metals. Ammonia can range from 12 to 50 mg/l and phosphorus can range from 6 to 20 mg/l in untreated sewage (Environmental Protection Agency, 1997)

Waste water treatment can involve physical, chemical or biological processes or combinations of these processes depending on the required outflow standards (EPA, 1997). A generalized layout of a waste water treatment plant is shown in figure 1.The first stage of waste water treatment takes place in the preliminary treatment plant where material such as oils, fats, grease, grit, rags and large solids are removed (cooper *etal*,1994). Primary settlement is sometimes used prior to biological treatment. Radial or horizontal flow tanks are normally employed to reduce the velocity of flow of the waste water such that a proportion of suspended matter settles out. Biological treatment of waste waters takes place in fixed media or suspended growth reactors using activated sludge, bio filtration, rotating biological contactors, constructed wetlands or variants of these processes. Nitrification/denitrification and biological phosphorus removal can be incorporated at this stage and will reduce nutrient concentrations in the outflow (cooper *et al*, 1994).

Chemical treatment is used to improve the settling abilities of suspended solids prior to a solid removal stage or to adjust the properties or components of waste water prior to biological treatment (e.g. pH adjustment, reduction of heavy metals or nutrient adjustment). It may also be used for precipitating phosphorus in conjunction with biological phosphorus treatment. Secondary settlement separates the sludge solids from the outflow of the biological stage. Tertiary treatment refers to processes which are used to further reduce parameter values below the standards set out in national regulations. The term is often used in reference to nutrient

removal. Sludge treatment can be a significant part of a waste water treatment plant and involves the stabilization and/or thickening and dewatering of sludge prior to reuse or disposal (EPA, 1995)

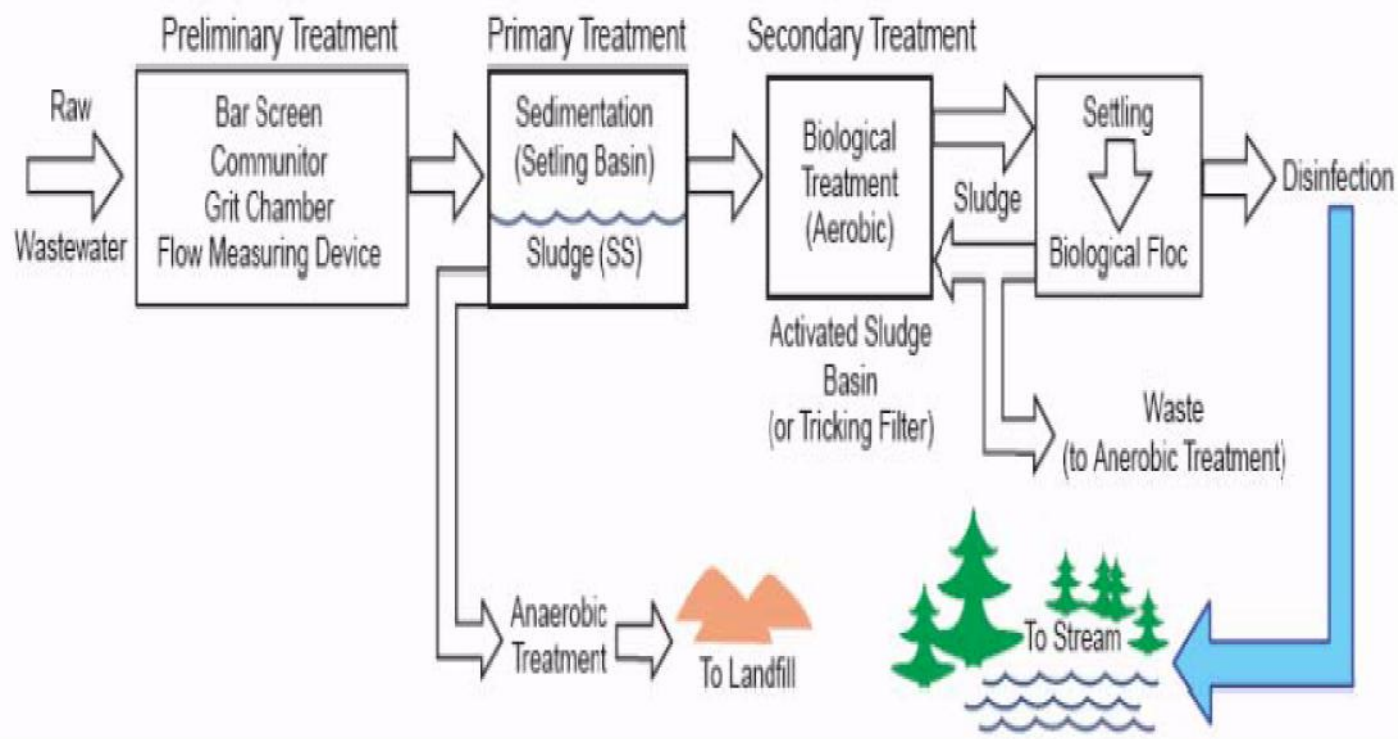


Figure 3. **:** Schematic of a typical wastewater treatment plant (Adopted from EPA, 1997)

2.3.Nature of Hospital wastewater

Waste water from hospitals is usually referred to as hospital waste and is defined as a special category of waste which comprises of all waste, biological or non-biological that is discarded from hospitals/health care centers and not intended for further use (Oyeleke *et al.* 2008). The important consumption of water in hospitals gives significant volumes of waste water loaded with microorganisms (the majority of which being pathogenic), heavy metals, toxic chemicals and radioactive elements. About 85% of hospital waste is said to be non-hazardous, 10% infective/hazardous and 5% not infective in the United States of America (Oyeleke and Istifanus, 2009).Whereas about 15% of hospital waste is regarded infective in most developed countries. This value could increase in India from 15% to 35% depending on the total amount of hospital waste generated (Agarwal, 1998), showing the impact of economic development on the quality of wastewater treatment.

Healthcare liquid waste is composed of residual quantity of disinfectant, antibiotics, other variety of chemicals, saprophytic microorganisms, commensal and pathogenic bacteria. Due to diverse interaction of these organisms (particularly pathogenic group) with themselves and chemical environment, there will be evolving of resistant bacteria that will pose difficulty for antibiotic treatment. Study conducted in Nepal found out that healthcare liquid wastes were loaded with multiple drug resistance bacteria that posed a huge public health threat in the transfer of such resistance to the bacterial pathogens causing community acquired infections, thereby limiting antibiotic pool (Aina *et al*, 2002). Also study conducted in Sweden demonstrated high prevalence of Vancomycin resistant *Enterococci* (VRE) in Swedish sewage, possibly due to antimicrobial drugs or chemicals released into the sewage system that may sustain VRE in the system (Prado *et al*, 2007)

The resistant bacteria isolated from wastes are diverse in nature. Study conducted in Nigeria showed that organisms belonging to seven genera of public health importance such as *Pseudomonas, Streptococcus, Serratia, Staphylococcus, Klebsiella, Proteus* and *Bacillus* showed varying degrees of resistance to the tested antimicrobial agents ranging from 0% to 77.8% (Sharma *et al*, 2010). Furthermore, the report explained, that among 25 organisms isolated from hospital A 16 phenotypic patterns of co-resistance to the tested disinfectants and antibiotics; were recognized; while from hospitals B and C 13 from among 18 and 9from among 14 isolates patterns were recognized, respectively, (Sharma *et al*., 2010)

Selection and dissemination of resistant bacteria in nature should be avoided in order to ensure effective treatment against infectious disease in humans and maintain an ecological balance that favors the predominance of a susceptible bacterial flora. Studies indicated difference in existence of antibiotic resistant bacteria in different sewages. For instance study conducted in Al-Shifa hospital, Gaza compared contribution of hospital wastewater to the spread of antibiotic resistance with non-health institution. The most frequently identified bacterium was *Pseudomonas sp*. (33.3% vs. 22.2%) followed by *E. coli* (31.9%vs.23.1%), *Enterococcus spp*. (36.4% vs. 21.2%), *Klebsiella sp.* (25% vs. 25) and *Proteus sp.* (28.6% vs. 23.8) Al-Shifa hospital and non-health institutions respectively. There was high incidence of antibiotic resistance among both gram-negative and gram-positive isolates and those isolated from

wastewater samples from Al-Shifa hospital and laboratory building of Islamic University of Gaza contained higher number of antibiotic resistant bacteria than bacteria isolated from other sites (Elmmanan*et al*, 2006).

2.4. Route of antimicrobial resistance from Hospital effluent to the environment

Antimicrobial resistance may spread in aquatic environment (drinking and recreational water) where its role is not restricted only as reservoir of clinical resistance genes, but also as a medium for spread and evolution of resistance genes and their vectors (Ekhaise and Omavwaya, 2008). Several investigations have reported data in support of this notion. For example, a study conducted in Brazil detected extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* in effluents and sludge of a hospital sewage treatment plant indicating that hospital wastewater treatment plant did not show a satisfactory efficacy in removing pathogenic micro-organisms, which allowed the dissemination of multidrug resistant bacteria into the environment (Reinthaler *et al*, 2003).

The presence of drug resistant bacteria in surface water and groundwater is a major public health concern as drug resistant bacteria could be transferred to humans via consumption of contaminated drinking water which then contributes to the spread and persistence of antibiotic resistance bacteria in the general population and the environment (Tao *et al*,2 010).

Untreated liquid hospital waste containing un-metabolized antibiotics in low concentration contributes largely to the development of antibiotic resistance in our natural micro flora/environmental micro flora (Schlusener and Bester, 2006). Antibiotics are poorly absorbed in human and animals gut with majority of them being excreted unchanged in faces and urine which eventually find their way into the environment through the disposal of sewage, hospital waste water and animal waste (Schlusener and Bester, 2006). Antibiotics used in hospitals and private households and released into effluent and municipal sewage indicates a selection pressure on bacteria (Kummerer and Henninger, 2003).

The influence of hospital wastewater containing resistant bacteria discharged to the receiving environment should not be underestimated. In this regard, public health impact is one of the most serious that requires urgent response from all stakeholders. One study conducted in Belgium to

compare the antimicrobial tolerance of Oxytetracycline-resistant heterotrophic bacteria isolated from hospital sewage and freshwater fish farm water generally showed that Oxytetracycline-resistant hospital heterotrophs displayed a higher frequency (84%) of Ampicillin (Amp) tolerance compared to the Oxytetracycline-resistant heterotrophs from the freshwater fish farm site (22%) (Huys *et al*, 2001).

The contribution of hospital waste water to the development of antimicrobial resistance is high and varies from species to species. The study conducted in Bangladesh to understand the role of untreated Hospital waste water in the emergence of multidrug resistance (MDR) bacteria showed that the total number of resistant bacteria found in different samples varied from site to site in respect to resistance against different number of antibiotic tested.(Rabbini *et al*, 2012). A Total of 59 *E. coli*, 29 *Klebsiella pneumoniae subsp. Pneumoniae, 3 Klebsiella pneumoniae subsp. Ozaenae* and 5 *Salmonella spp.* isolates were recovered from waste water sample. Resistance among *E. coli and Klebsiella pneumoniae subsp. Pneumoniae* were Erythromycin 100 and 100%; Penicillin 100 and 100%; Azithromycin 97 and 97%; Cefotaxime 48 and 45%; Ceftazidime 40 and 38%; Ampicillin 71 and 100%; Streptomycin 50 and 34%; Sulfamethoxazole-trimethoprim 58 and 28%; Ciprofloxacin 50 and 17%; Kanamycin 38 and 10%; Chloramphenicol 28 and 10%; Gentamicin 19 and 16% and Imipenem 7% and 0%; respectively (Rabbini*et al*, 2012)

Studies have demonstrated that hospital wastewater is highly selective environments and that they contribute to the high rates of resistant bacteria that are being discharged in the natural environment (Yang *et al*, 2009). The occurrence of bacteriophages from samples of animals fecal wastes can be environmental vectors for the horizontal transfer of antibiotic resistance genes(Colomer-Lluch *et al*, 2011). Therefore, antibiotic resistance is not only found in pathogenic bacteria but also in environmental organisms inhabiting terrestrial and aquatic habitats. Higher numbers of resistant bacteria occur in polluted habitats compared with unpolluted habitats, indicating that humans have contributed substantially to the increased proportion of resistant bacteria occurring in the environment (Pathak *et al*, 1993).

Antibiotics exert selection in favor of resistant bacteria by killing or inhibiting growth of susceptible bacteria (Figure 2); resistant bacteria can adapt to environmental conditions and serve as vectors for the spread of antibiotic resistance (Kruse, 1999).Waste effluent from hospitals

contains high numbers of resistant bacteria and antibiotic residues at concentrations able to inhibit the growth of susceptible bacteria (Grabow and Prozesky, 1973). As a result, hospital waste effluent could increase the numbers of resistant bacteria in the recipient sewers by both mechanisms of introduction and selection for resistant bacteria (Al-Ahmed *et al*, 1999).The main risk for public health is that resistance genes are transferred from environmental bacteria to human pathogen (Kruse, 1999; Wegener *et al*, 1999).

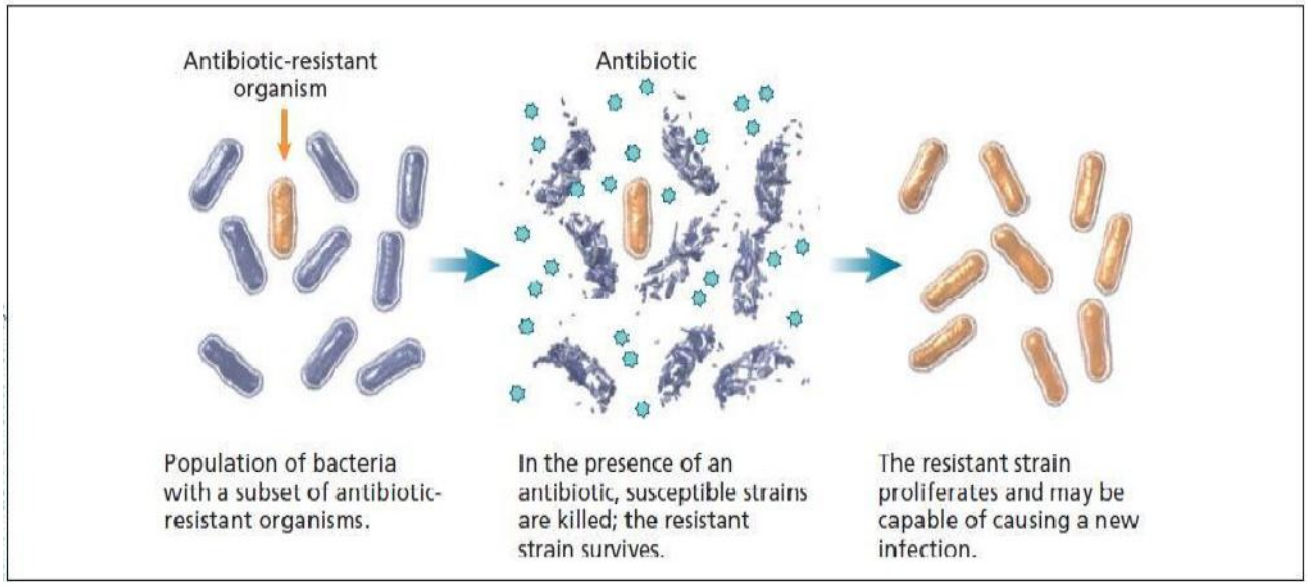


Figure 4. Effect of selective antibiotic pressure on bacteria **(**Adopted from Rabbani*et al*, 2012).

2.5.Effect of Hospital wastewater on public sewage and beyond

Small amounts of important chemicals are regularly discharged from hospitals into sewers; that include anesthetics, disinfectants (formaldehyde, glutaraldehyde), chemicals from laboratory activities, photochemical solutions (hydroquinone), and X-ray contrast media containing absorbable organohalogen compounds (AOX),Mercury from dental amalgams or lab chemicals, excessive nutrients and nitrates(Focazio *et al*, 2008). Small quantities of pharmaceuticals including antibiotics and genotoxic drugs are usually discharged to the sewers from hospital pharmacies and from the various wards. Pharmaceuticals in water may act as endocrine disruptors and could result in antibiotic-resistant pathogens (Ruhoy and Daughtou, 2008)

In developed countries, water use is commonly high and the sewage therefore greatly diluted; effluents are treated in municipal treatment plants and no significant health risks should be expected, even without further specific treatment of these effluents. Only in the unlikely event of an outbreak of acute diarrheal diseases should excreta from patients be collected separately and disinfected. In developing countries, where there may be no connection to municipal sewage networks, discharge of untreated or inadequately treated sewage to the environment will inevitably pose major health risks (Mara and Cairncross, 1989).

Wastewater is potentially infectious, some healthcare facilities in low-income areas have no sewer systems and some are not watertight, and wastewater can leak into groundwater .Improper wastewater management, collection, treatment, and disposal may result in the pollution of local drinking water sources, or the contamination of natural resources. Excessive nutrients cause biological degradation in groundwater, lakes and rivers by using up oxygen (eutrophication) resulting in algal blooms and bio toxins (Abu-Amar and Yassin, 2008). The principal area of concern is wastewater with a high content of enteric pathogens, including bacteria, viruses, and helminthes, which are easily transmitted through water. Contaminated wastewater is produced by wards treating patients with enteric diseases and is a particular problem during outbreaks of diarrheal disease (Schets *et al*, 2008).

A study conducted in Nigeria demonstrated that hospital wastewater was observed to play a significant role in influencing the qualities of the bacteriological and physiochemical parameters on the receiving environment due to increased amount of organic matter and essential nutrients in hospital wastewater (Mesdaghinia *et al*, 2009). Most of the time hospital effluents are released with other sewage to water bodies (lakes, rivers, streams, or underground water) which will be used as medium for transfer of resistant bacteria and their genes in aquatic system. As these water bodies are one of the major sources of water, directly or indirectly, for human and animal consumption, its pollution may contribute to the maintenance and even the spread of bacterial antibiotic resistance (Ekhaise and Omavwaya, 2008)

Hazardous medical waste consists primarily of chemicals and discarded cytotoxic drugs which find their way into the environment due to improper usage and indiscriminate disposal. Their presence in the environment have been reported to pose serious environmental health risk due to their toxic, genotoxic and/or carcinogenic effect (Akter *et al.* 1999;Shaner, 1997) which as a

result could have potential negative effects on the biological balance of natural environment. Environments like clinical settings frequently contain elevated levels of antibiotics and other pharmaceuticals. Consequently they are considered to select for antibiotic resistance and to be important hot spots for horizontal gene transfer (HGT) of resistance genes, and thus sites of resistance evolution (Baquero *et al*,2008). Most of the microorganisms in hospital waste water have also been reported to be resistant to the commonly used antibiotics and as such have led to the outbreak of several diseases/infections (Chitnis *et al*, 2000).

2.6.Status of antimicrobial resistant pathogens in Hospital wastewater in Ethiopia

In developed countries domestic wastewater and Hospital effluents are discharged, usually, in the urban sewer system where they mix with other effluents and finally reach the sewage treatment plant. The last step of this process is the release of purified wastewaters to a river, a lake, or seawaters. Some of these water bodies may serve as sources of drinking water at somewhere in the community. As a result, dissemination of antimicrobial resistance bacteria in the environment will be minimized. This problem in developed country less severe compared to developing country, mainly due to proper antibiotic usage, effective infection control program and better management of hospital wastewater. The situation in developing country, like Ethiopia is overwhelming as the above factors are less practiced. In addition to this most hospitals in Ethiopia neither have hospital wastewater treatment plant nor discharge waste to urban sewer system, which worsen the problem.

Antibiotic resistance pattern to common antibiotics in Hospital effluents are not studied well in Ethiopia to update Hospital effluent management and proper antibiotic usage. Only few researches by Ethiopian researchers were conducted in this regard. For example, the study carried out in Southern part of Ethiopia that assessed disinfectant and antibiotic resistance bacteria in Hospital waste water at Yirgalem Hospital(YAH) and Hawassa University referral Hospital(HURH) showed that pathogenic (*Salmonella, Shigella and S. aureus*) and potentially pathogenic (*E. coli)* bacteria were detected from effluents of both hospitals (Fekadu *et al*, 2015). *Salmonella* isolates from YAH effluent were resistant to Ceftriaxone, Tetracycline and Doxycycline, whereas from HURH effluent the isolates were resistant to Gentamycin in addition to the above three antibiotics. Similarly, while S. aureus from YAH effluent was resistant to

Penicillin, Ampicillin and Amoxicillin, isolates from HURH were resistant to Gentamycin in addition to the above three antibiotics (Fekadu *et al*, 2015).According to this study High numbers of indicator organisms were obtained from effluents of YAH, although microbiological indicators exceed WHO standard for both hospital effluents. This is an indication for possible presence of pathogenic organisms which is discharged in to receiving environment (lake and river) posing risk to public health too and this study revealed the similarity in antimicrobial resistance pattern in both hospital effluents which warrants for appropriate interventions(Fekadu *et al*, 2015)

Another study conducted in Northwest Ethiopia with aims of isolation and characterization of multiple drug resistance bacterial pathogens from waste water in hospital and non-hospital environments indicated that a total of 60 waste water samples were processed for the presence of drug resistance pathogens. Among the total samples 113 bacterial isolates were recovered and of these 65 (57.5%) were from hospital environment and 48 (42.5%) were from non-hospital environment. The most frequently identified bacterium was *Klebsiella spp.*30 (26.6%) followed by *Pseudomonas spp.* 19(16.8%), *E. coli* (11 .5%) and *Citrobacter spp.* (11.5%), and *Staphylococcus aureus* (8.2%). The overall prevalence of multiple drug resistance (MDR) in this study was 79/113 (69.9%). MDR in hospital environment was found to be 53/68 (81.5%) while in non-hospital environment was found to be 26/48(54.2 % (Moges *et al*, 2014). Based on this study multiple drug resistance to the commonly used antibiotics was high in the study area. This was even higher in hospital waste water. The contamination of water by antibiotics or other pollutants lead to the rise of resistance due to selection pressure. The presence of antibiotic resistant organisms in this waste water should not be overlooked. Since this organisms may be vital to the safety and well-being of patients who are hospitalized as well as individuals who are susceptible to infection (Moges *et al*, 2014)

Continued updating of the susceptibility pattern of recent clinical isolates from hospital wastewater and periodic follow-up in changes of antibiotic patterns are necessary in every country. This is particularly important to prevent population of bacteria resistant to antibiotics and select antibiotic of choice for particular condition.

3. Objectives of the study

3.1.General objective

To isolate common bacterial pathogens and assess antimicrobial resistance of bacteria isolated from sewage released from selected hospitals and non-hospital environments in Addis Ababa, Ethiopia

3.2. Specific objectives

- To isolate selected common pathogenic and potentially pathogenic bacteria from hospital and non-hospital sewage (*E.coli*, *Klebsiella*, *Citrobacter*, *Pseudomonas* and *staphylococcus spp.)*
- To assess drug resistance patterns of the bacterial isolates to commonly used antibiotics
- To compare antimicrobial drug resistance pattern of bacteria isolated from hospital wastewater with that isolated from non-hospital waste water.

4. Methods and materials

4.1.Study area and period

The study was conducted in Addis Ababa one tertiary and three referral hospitals. These hospitals were selected by convenience because of their accessibility to take sample as determined from situation analysis made during field visit in advance, and because of number of population served by these hospitals. The tertiary hospital was Tikure Anbessa specialized hospital while the 3 referral hospitals were Minilik II, Ras Desta Damtew Memorial and Zewditu Memorial referral hospitals. These hospitals provide a range of health services to the population of Addis Ababa region and for the people referred from different parts of the country. Tertiary and referral hospitals are meant to provide health services for 3.5 up to 5 million people and 1.5 million people, respectively (FMOH, 2010)

A filed visit to these hospitals showed at the time none of these hospitals to have sewage treatment plant; even some of them were without properly working septic tank .Some of these hospitals release their waste to the municipal sewerage system directly, which will finally be joining rivers after going long distance; some dispose sewage by long distance tracks; and others release directly to rivers without proper treatment. Sewage from 4 randomly selected non-hospital sources was also included in the study. The study was conducted from August_2016-December_2017.

4.2.Study design

A cross sectional study design was used to conduct the research and hospital and non-hospital sewage samples were collected at different times points of a day during the study period (within the 5 weeks study period).

4.3. Sample size and sampling technique

A total of two hundred and twenty sewage samples from 4 selected hospitals and 4 non-hospital sites (1 site in close proximity to each hospital sites) were collected.

From each hospital site one, two or three (based on accessibility of the site to collect sample) waste water samples were collected twice a week at two time points for five weeks (10:30 am & 2:30 pm) as shown below:

1. Before entering into the septic tank (Influent sample),
2. From septic tank and
3. Before released into the environment (effluent sample)

Non-clinical wastewater samples were collected, as shown in table 1 below, twice a week at two different time points a day (10:30 am & 2:30 pm) from each of the following 4sites; (Zewditu Memorial Hospital Administration building; Tikure Anbessa Specialized hospital_medical students dormitory; Minilik II referral hospital health science college building and Ras Desta Damtew Memorial hospital administration building).Thus, the numbers of wastewater samples studied were

Table 1. The sources of waste water samples and numbers of samples collected

Sources of wastewater samples		**Number of sampling places within the sample source**	**Frequency of sample collection during each sampling day**	**Number of sampling weeks X Frequency of sampling per week**	**Total number of samples collected from a single source**
Hospital wastewater sources **(N=140)**	TikureAnbessa Specialized Hospital	1	2	5 X 2 =10	20
	Zewditu Memorial Hospital	1	2	5 X 2 =10	20
	Menilik II Referral Hospital	2	2	5 X 2 =10	40
	RasDestaDamtew Memorial Hospital	3	2	5 X 2 =10	60
Non-Hospital (non-clinical) wastewater sources **(N=80)**	Non-clinical Site 1	1	2	5 X 2= 10	20
	Non-clinical Site 2	1	2	5 X 2= 10	20
	Non-clinical Site 3	1	2	5 X 2= 10	20
	Non-clinical Site 4	1	2	5 X 2= 10	20

Non-clinical Site: 1. TikureAnbessa Specialized hospital medical students' dormitory
Non-clinical Site: 2: Zewditu Memorial Hospital administration building
Non-clinical Site: 3 Minilik II referral hospital health science college building
Non-clinical Site: 4 RasDestaDamtew hospital administration building

4.3.1 Sample collection, transportation and storage

Each sample was collected in sterile bottle of 500 mL and then transported in ice jackets with ice box to Addis Ababa University Health Science College's microbiology teaching laboratory for bacteriological analysis. Then it was stored in refrigerator at 4ºC until analysis. All the samples were analyzed on the day of collection.

4.4.Laboratory methods

4.4.1. Culture

The sample was thoroughly shaken to get homogenous mixture before a portion was taken for culture. Standard procedures were followed in all bacteriological analyses. Serial 10-fold dilutions of wastewater samples were prepared in 0.85% NaCl(normal saline). Serial dilution was done based on the principle that when soil sample or water sample along with bacterial colonies are taken, the result obtained in the form of reduced bacterial colonies would be more appropriate in order to get pure colonies. One ml of each sample was poured into nine ml of sterile normal saline and serially diluted from one tube (containing 9ml sterile normal saline) to the next in a 10-fold dilution until 8^{th} fold dilution was reached. The bacteria were cultivated by plating 0.5ml each of the desired serial dilutions of the bacterial suspensions, i.e., the 5th and 6th ($10^{-5}$and 10^{-6}) dilutions of non-hospital and the 7th and 8th ($10^{-7}$and 10^{-8}) dilutions of hospital waste water. Duplicate samples were plated onto MacConkey (MAC) agar, Mannitol Salt Agar (MSA) and pseudomonas agar and incubated at 37ºc for 24-48 hours. Identification of most important pathogenic bacteria found in hospital and non-hospital sewage was done based on their colony appearance, gram staining, growth on selective media and biochemical test according to the standard methods for examination of water and wastewater (American Public Health association, 1999).Pure colonies were prepared by sub culturing on MacConkey (MAC) agar, blood agar (BA), Mannitol Salt Agar (MSA) and pseudomonas agar. After obtaining pure colonies and recording important features, isolated organisms were further identified to the species level biochemically following standard methods (Vandepitte, 1996)

4.4.2. Biochemical Testing

Initially gram positive bacteria were separated from gram negative ones using gram staining. Then colony morphology was used to pick up isolates suspected to belong to different genera. Suspected *E. coli* appears on MacConkey agar as a dry, flat, pink colony, whereas *Klebsiella spp.* colonies are shiny, —mucous-like texture and have a darker pink center; *Enterobacter spp*. form pink coloured mucoid colonies; on the other hand, *Citrobacter spp* .colonies have smooth pale to pink coloured appearance (Chessbrough M, 2006).*E. coli*, *Klebsiella spp.*, *Enterobacter spp*.and *Citrobacter spp.* were separated from each other using seven tests: indole, urease production, manitol, citrate utilization, H_2S production, motility, and LDC reactions. Bacterial isolates were identified as *E. coli* with the following biochemical test results: indole positive, urea negative, manitol positive, H_2S negative with TSI test, citrate negative, motility negative and LDC reactions positive. Likewise, *Klebsiella spp.* identification was made using same tests: indole negative, urea positive, manitol positive, H_2S negative with TSI test, citrate positive, motility negative and LDC reactions negative. *Enterobacter spp*. were identified biochemically as follows: indole negative, urea test negative, manitol positive, H_2S negative with TSI test, citrate positive, motility positive and LDC reactions positive. For *Citrobacter spp*. biochemical test results were: indole negative, urea test negative, manitol positive, H_2S positive with TSI test, citrate positive, motility positive and LDC reactions negative. *Pseudomonas* on *pseudomonas* agar plate forms blue-green or brown pigment and also further confirmed by positive oxidase and catalase reactions from blood agar. *S.aureus* colonies on mannitol salt agar (MSA) appear yellow and are gram positive with grape morphology under the microscope. In addition, they were positive for both catalase and Coagulase slide tests. .

4.4.3. Antimicrobial susceptibility testing

Once the bacteria were isolated and identified from each collected sample, the standard Kirby - Bauer disk diffusion method was used to determine the antimicrobial susceptibility profiles of the isolates (Bauer *et al*, 1966). Bacterial inoculum was prepared by suspending the freshly grown bacteria in 4–5 ml normal saline and the turbidity was adjusted to that of a 0.5 McFarland standard. Then this suspension was spread on over the entire surface of the Mueller-Hinton agar using cotton swab to produce confluent growth. Paper discs impregnated with specific amounts

of commonly prescribed antimicrobial agents for inpatient use were then placed on the surface of the medium, and aerobically incubated at 37°C for 18-24 hours. Discs of the following antimicrobial agents were tested: Ceftriaxone (30µg), Ampicillin (10µg), Amoxicillin (25µg), Ciprofloxacin (5µg), Imipenem (µg10), Cefotaxim (30µg), Chloramphenicol (30µg), Kanamycin (30µg),Ceftazidime (30µg), Gentamycin (10µg) and Erythromycin (15µg). The zones of inhibition were measured and compared with National Committee for Clinical Laboratory Standards (N CCL S) guidelines (Wayne, 2000).

4.5.Quality control

Qualities of data obtained were insured by following standard procedure in each step of the work. The functionality of instruments was checked before employing for the process. The quality of media, reagents, stains and antibiotic disks were insured following the manufacturer's direction. In addition, the following reference strains (kindly donated by EPHI) were used to control the performance of disk diffusion test and biochemical tests: *Pseudomonas aeruginosa*(27853*), Escherichia coli* (25922) and *Staphylococcus aureus* (25923) according to CLSI recommendation.

4.6.Data analysis

Data was entered, cleaned and analyzed using SPSS Statistical Software version 20(IBM company, Comp.soft-sys.stat.spss.). Descriptive statistics was employed to report numerical summaries of findings. Patterns of quantitative values were presented using graph presentations and statistical tables. One way ANOVAs, independent students' t test and paired t test were used to compare means of some parameters. A critical value of 0.05 was used for the inferential statistics.

4.7.Ethical considerations

The proposal was evaluated by Department of medical Microbiology, Immunology and parasitology (DMIP) ethical committee of the Medical School of AAU and institution review board (IRB) before the start of the research. Letters of request for cooperation were obtained from Department to hospitals where samples were collected in order to get permission.

5. Results

5.1. Isolation of pathogenic/potentially pathogenic bacteria

A total of 220 samples were collected twice a week at two time points per day for a period of five weeks for microbiological analysis and the presence of drug resistance bacterial pathogens. All samples were preserved at 4ºC in refrigerator until analysis and processed on the day of collection. Of these samples 210(95.45%) [134(95.7%) from hospital environment; and 76(95%) from non-hospital environment] were culture-positive which gave rise to one or more bacterial isolates. A total of 506 bacterial isolates were obtained, 327 (64.62%) from hospital environment and 179 (35.37%) from non-hospital environment as shown in the (Table 2) below

Table2.Distribution of bacterial culture positive and negative samples taken from hospital and non-hospital environments, Addis Ababa 2016/17

Sample sites	Total Samples N (%)	Sample positive N (%)	Sample Negative N (%)	Total bacteria isolates Recovered N (%)
Hospital wastewater				
TASH	20(100)	20(100)	0	55(16.8)
ZMH	20(100)	16(80)	4(20)	43(13.15)
RDDMH	60(100)	58(96.6)	2(3.33)	131(40.06)
MIIRH	40 (100)	40(100)	0	98(29.96)
Sub total	**140(100)**	134(95.7)	6(4.3)	**327(64.62)**
Non-hospital wastewater				
ZMH administration Building	20(100)	20(100)	0	40(22.35)
MIIRH health science collage building	20(100)	20(100)	0	45(25.14)
RDDMH administration Building	20(100)	20(100)	0	50(27.93)
BLH CHS medical students dormitory building	20(100)	16(80)	4(20)	44(24.58)
Sub total	**80(100)**	76(95)	4(5)	**179(35.35)**
Over all	**220(100)**	210(95.45)	10(4.54)	**506(100)**

TASH–TikureAnbessa Specialized Hospital; **ZMH**- Zewditu Memorial Hospital; **RDDMH**- RasDestaDamtew Memorial Hospital; **MIIRH**- Minillik 2nd Referral Hospital

The most frequently isolated bacterium from both hospital and non-hospital environments was *pseudomonas spp.*160 (31.8%) followed by *E.coli* 108(21.5%), *Klebsiellaspp.* 76(15%),*Citrobacterspp*.50(10%),*S. aureus* 37(7.4%) and *Enterobacter* 14(2.8%)(Table 3)

Table 3.Number and types of bacterial isolates at each sampling site from hospital and non-hospital wastewater sample Addis Ababa, 2016/17

Bacterial isolates	Hospital wastewater No. (%)	Non- hospital wastewater No. (%)	Total No. (%)
Pseudomonas spp.	112(34.25)	48(26.8)	160(31.62)
E.coli	62(18.96)	46(25.69)	108(21.34)
Klebsiella spp.	62(18.96)	14(7.8)	76(15.01)
Citrobacter spp.	24(7.34)	26((14.5)	50(9.88)
Enterobacter	6(1.8)	8(4.46)	14(2.76)
S.aureus	25(7.6)	12(6.7)	37(7.3)
CONS*	18(5.5)	11(6.14)	29(5.7)
Others**	18(5.5)	14(7.8)	32(6.3)
Total	327(100)	179(100)	506(100)

*CONS-coagulase negative *Staphylococci*

**Others-*Serratia*(16 isolates)-*Providencia*(6 isolates)- *Acinetobacter*(6 isolates)

*Proteus(*4 isolates)

From the total 140 wastewater samples collected from the four hospitals (Ras Desta Damtew Memorial, Minilik Π Referral, Zewditu Memorial and Tikure Anbessa Specialized Hospitals), 327 bacterial strains were isolated. Ras Desta Damtew Memorial hospital accounted for 134(40.97%) of isolates followed by Minilik Π Referral Hospital for 101(30.9%) isolates, Black Lion specialized hospital for 53(16.2%) and Zewditu Memorial Hospital for 39(11.93%).As the numbers of samples collected from the four hospitals differed (except TASH and ZMH, which had the same sample size), so also did numbers of isolated bacteria differ between hospitals; some isolates like *Providencia, Proteus* and *Acinetobacter* were isolated from only TASH, RDDMH and MIIRH, respectively. There was no *Citrobacter spp*. isolated from wastewater samples collected from TASH. Wastewater samples were collected

in two different time points (10:30am & 2:30pm); but there was no significant difference between types and numbers of bacteria isolated between these time points. Also wastewater samples were collected from different places with in the hospital (influent, septic tank and effluent); there were some notable differences in number of isolates between places in RDDMH isolates. Some isolates were found in the influent samples only, but some were in the septic tank or effluent samples(Table 4)

Table 4. Number of bacteria isolated from each sampling point of the four Hospitals in Addis Ababa, 2016/17

Isolated Bacteria	Sampling sites														
	RDDMH						**M ΠRH**				**TASH**		**ZMH**		Total
	Influent		Septic tank		effluent		Septic tank		Effluent		Effluent		Effluent		
	A	P	A	P	A	P	A	P	A	P	A	P	A	P	
Pseudomonas	11	9	10	6	5	7	9	7	7	5	7	9	9	11	112
Klebsiella	8	12	2	2	3	1	3	5	3	5	6	8	3	1	62
E. coli	5	3	1	3	6	6	7	5	8	4	5	5	2	2	62
Citrobacter	2	-	1	1	3	1	1	3	2	6	-	-	1	3	24
Entrobacter	-	-	2	-	-	2	-	-	-	-	-	2	-	-	6
Serattia	-	-	-	-	2	2	-	-	3	1	-	-	-	-	8
Acinetobacter	-	-	-	-	-	-	2	2	2	-	-	-	-	-	6
Providencia	-	-	-	-	-	-	-	-	-	-	1	1	-	-	2
Proteus	-	2	-	-	-	-	-	-	-	-	-	-	-	-	2
CONS	2	1	-	2	1	1	2	1	-	2	2	1	2	1	18
S.aureus	2	2	2	1	2	-	2	2	1	1	4	2	2	2	25
Total	30	29	18	15	22	20	26	25	26	24	25	28	19	20	327

KeyA= samples collected at 10:30am ZMH= Zewditu Memorial Hospital

P= samples collected at 2:30pm TASH= Tikure Anbessa Specialized Hospital

RDDMH= Ras Desta Damtew Memorial Hospital

M ΠRH=Minilik Second Referral Hospital

A total of 179 bacterial strains were isolated from 80 wastewater samples collected from four non- hospital sites. Bacteria isolated from RDDMH administration building accounted for 51(28.5%) of isolates followed; MIIRH health science college building, 46(25.7%);TASH CHS medical students dormitory building, 42(23.5%); and ZMH administration Building, 40(22.3%).There was no significant difference between numbers and types of bacteria isolated in different time points (10:30am & 2:30pm) from these four non-hospital sites (Table 5)

Table 5. Number of bacteria isolated from each sampling point of the four Non- Hospital sites in Addis Ababa, 2016/17

Isolated Bacteria	Sampling sites								
	Non-hospital site 4		Non-hospital site 3		Non-hospital site 1		Non-hospital site 2		Total
	Effluent		Effluent		Effluent		Effluent		
	A	P	A	P	A	P	A	P	
Pseudomonas	6	4	5	5	11	9	3	5	48
Klebsiella	2	4	5	3	-	-	-	-	14
E. coli	3	7	4	4	5	7	9	7	46
Citrobacter	4	4	6	4	3	1	2	2	26
Entrobacter	2	-	2	-	-	-	1	3	8
Serattia	2	2	2	-	-	2	-	-	8
Acinetobacter	2		-	-	-	-	-	-	2
Providencia	-		-		-		3	1	4
CONS	3	1	1	2	2	-	1	1	11
S.aureus	3	2	2	1	2	-	2	-	12
Total	27	24	27	19	23	19	21	19	179

Non-hospital site 1= TASH CHS medical students dormitory building

Non-hospital site 2= ZMH administration Building

Non-hospital site 3= MIIRH health science collage building

Non-hospital site 4= RDDMH administration Building

A-samples collected at 10:30am; P-samples collected at 2:30pm

5.2. Drug susceptibility pattern

Drug Susceptibility of medically important bacteria (*Pseudomonas, Klebsiella, E. coli, Citrobacter and S.aureus*) isolated from the four hospitals and non-hospital wastewater samples to commonly prescribed antibiotics was determined using Kirby-Bauer disk diffusion methods. The zone of inhibitions were measured in millimeters and interpreted as susceptible, intermediate or resistant according to susceptibility breakpoints of national committee for Clinical and Laboratory Standards Institute (CLSI). Patterns of susceptibility to different antibiotics are shown below in the tables6,7,8 9 and 10

Multiple drug resistance was common among *Pseudomonas spp*. isolates to commonly used antibiotics in the study area. All the isolates of four hospitals were 100% resistant to Ampicillin and Cefotaxim followed by Ceftazidim(96.4%), Ceftriaxone (82.1%), Ciprofloxacin (35.7%), Chloramphenicol and Kanamycin (26.8% each); and leastresistance was observed against Gentamicin (17.8%) and Imipenem (100% susceptible).(Table 6)

Drug resistance rates for *Pseudomonas spp***.** against the following drugs were:- Ceftriaxone (91.1%, 80%, 75% and 71.4%),Chloramphenicol (58.3%, 0%, (12.5%) and 0%), Ciprofloxacin (50%), (40%), (25%) and (14.3%), Gentamicin (33.3%), (0%), (0%) and (14.3%) and Kanamycin (41.7%, 20%, 12.5% and 14.3%), respectively for RDDMH, ZMH, TASH and MIIRH.RDDMH isolates showed highest resistance rates; there was statistically significant difference between RDDMH and other hospitals ($p<0.01$)(Table 6)

Table 6. Drug susceptibility pattern of ***Pseudomonas spp.*** Isolates from hospital wastewater

Antibiotics	Susceptibility pattern	Sample source				Total count (%)	P
		RDDMH	М ПRH	TASH	ZMH		
Ampicillin(10 µg)	S N (%)	0	0	0	0	0	
	I N (%)	0	0	0	0	0	
	R N (%)	48(100)	28(100)	16(100)	20(100)	112(100	NS
Cefotaxim(30 µg)	S N (%)	0	0	0	0	0	
	I N (%)	0	0	0	0	0	
	R N (%)	48(100)	28(100)	16(100)	20(100)	112(100)	NS
Ceftazidim(30 µg)	S N (%)	0	0	0	0	0	NS
	I N (%)	0	4(14)	0	0	4(3.6)	
	R N (%)	48(100)	24(86)	16(100)	20(100)	108(96.4)	
Ceftriaxone(30 µg)	S N (%)	4(8.3)	8(28.6)	2(12.5)	4(20)	18(16.1)	
	I N (%)	0	0	2(12.5)	0	2(1.8)	<0.01
	R N (%)	44(91.7)	20(71.4)	12(75)	16(80)	92(82.1)	
Chloramphenicol(30 µg)	S N (%)	8(16.7)	24(85.7)	12(75)	12(60)	56(50)	
	I N (%)	12(25)	4(14.3)	2(12.5)	8(40)	26(23.2)	<0.001
	R N (%)	28(58.3)		2(12.5)		30(26.8)	
Ciprofloxacin(5 µg)	S N (%)	16(33.3)	20(71.)	4(25)	12(60)	52(46.4)	
	I N (%)	8(16.7)	4(14.3)	8(50)		20(17.9)	<0.001
	R N (%)	24(50)	4(14.3)	4(25)	8(40)	40(35.7)	
Gentamicin(10 µg)	S N (%)	28(58.3)	24(85.)	16(100)	20(10)	88(78.6)	
	I N (%)	4(8.3)	0	0	0	4(3.6)	<0.001
	R N (%)	16(33.3)	4(14.3)	0	0	20(17.8)	
Imipenem(10 µg)	S N (%)	48(100)	28(100)	16(100)	20(100)	112(100)	NS
	I N (%)	0	0	0	0	0	
	R N (%)	0	0	0	0	0	
Kanamycin(30µg)	S N (%)	16(33.3)	20(71.4)	10(62.5)	8(40)	54(48.2)	
	I N (%)	12(25)	4(14.3)	4(25)	8(40)	28(25)	<0.001
	R N (%)	20(41.7)	4(14.3)	2(12.5)	4(20)	30(26.8)	

P=p-value NS- no statistical difference

Nearly all the isolates of *Klebsiella spp.* were resistant to Ampicillin and Cefotaxim (100%). No isolates were resistant to Imipenem (100% susceptible)*Klebsiella* isolated from TASH showed highest resistance rates against: Ceftriaxone, Ciprofloxacin and

Kanamycin. The result showed statistically significant difference between TASH isolates and isolates from other hospitals ($p<0.001$); МПRH isolates showed highest resistance rate against Chloramphenicol(that was statistically significant difference($p<0.001$)

Table 7. Drug susceptibility patterns of ***Klebsiella spp.*** isolates from hospital wastewater

Antibiotics	Susceptibility pattern	Sample source				Total count (%)	P
		RDDMH	M ПRH	TASH	ZMH		
Ampicillin(10µg)	S N (%)	0	0	0	0	0	
	I N (%)	0	0	0	0	0	
	R N (%)	28(100)	16(100)	14(100)	4(100)	62(100)	NS
Cefotaxim(30µg)	S N (%)	0	0	0	0	0	
	I N (%)	0	0	0	0	0	
	R N (%)	28(100)	16(100)	14(100)	4(100)	62(100)	NS
Ceftazidim(30µg)	S N (%)	0	0	0	0	0	NS
	I N (%)	0	0	2(14.3)	0	2(3.2)	
	R N (%)	28(100)	16(100)	12(85.7)	4(100)	60(96.8)	
Ceftriaxone(30µg)	S N (%)		8(50)			8(12.9)	
	I N (%)	8(28.6)	4(25)		1(25)	13(21)	<0.001
	R N (%)	20(71.4)	4(25)	14(100)	3(75)	41(66.1)	
Chloramphenicol(30µg)	S N (%)	28(100)	12(75)	14(100)	4(100)	58(93.5)	<0.001
	I N (%)	0	0	0	0	0	
	R N (%)	0	4(25)	0	0	4(6.5)	
Ciprofloxacin(5µg)	S N (%)	20(71.4)	8(50)	4(28.6)	2(50)	34(54.8)	
	I N (%)	8(28.6)	4(25)	2(14.3)	1(25)	15(24.2)	<0.001
	R N (%)		4(25)	8(57.1)	1(25)	13(21)	
Gentamicin(10µg)	S N (%)	28(100)	12(75)	12(85.7)	3(75)	55(88.7)	<0.001
	I N (%)	0	0	0	0	0	
	R N (%)	0	4(25)	2(14.3)	1(25)	7(11.3)	
Imipenem(10µg)	S N (%)	28(100)	16(100)	14(100)	4(100)	62(100)	NS
	I N (%)	0	0	0	0	0	
	R N (%)	0	0	0	0	0	
Kanamycin(30µg)	S N (%)	8(100)	12(75)	4(28.6)	2(50)	46(74.2)	
	I N (%)	0	4(25)	2(14.3)	1(25)	7(11.3)	<0.001
	R N (%)	0	0	8(57.1)	1(25)	9(14.5)	

P= p-value NS-no statistical significance

All the isolates of *E.coli spp.* from hospital wastewater were resistant to Ampicillin and

Cefotaxim (100%), followed by Ceftazidim (82.2%). All the isolates were 100% susceptible to Imipenem followed by Chloramphenicol and Gentamicin (sensitivity of 80.6% and 77.4%, respectively). *E.coli* isolated from RDDMH showed highest resistance ratesfor:Ceftazidim(100%),Ceftriaxone(100%),Chloramphenicol(50%),Ciprofloxacin(83.3%),Gentamicin(50%) and Kanamycin(66.7%).There was statistically significant difference (P<0.01)between RDDMH isolates and isolates from other hospital (Table 8)

Table 8. Drug susceptibility patterns of ***E.coli spp.*** isolates from hospital wastewater

Antibiotics	Susceptibility pattern		Sample source				Total count (%)	P
			RDDMH	М ПRH	TASH	ZMH		
Ampicillin(10µg)	S	N (%)	0	0	0	0	0	
	I	N (%)	0	0	0	0	0	
	R	N (%)	24(100)	24(100)	10(100)	4(100)	62(100)	NS
Cefotaxim(30µg)	S	N (%)	0	0	0	0	0	
	I	N (%)	0	0	0	0	0	
	R	N (%)	24(100)	24(100)	10(100)	4(100)	62(100)	NS
Ceftazidim(30µg)	S	N (%)	0	0	0	0	0	
	I	N (%)	0	4(16.7)	6(60)	1(25)	11(17.8)	<0.01
	R	N (%)	24(100)	20(83.3)	4(40)	3(75)	51(82.2)	
Ceftriaxone(30µg)	S	N (%)		4(16.7)	6(60)	2(50)	12(19.3)	
	I	N (%)		8(33.3)			8(12.9)	<0.0001
	R	N (%)	24(100)	12(50)	4(40)	2(50)	42(67.8)	
Chloramphenicol(30µg)	S	N (%)	12(50)	24(100)	10(100)	4(100)	50(80.6)	
	R	N (%)	12(50)				12(19.4)	<0.0001
Ciprofloxacin(5µg)	S	N (%)	4(16.7)	12(50)	8(80)	3(75)	27(43.5)	
	I	N (%)		8(33.3)			8(12.9)	<0.0001
	R	N (%)	20(83.3)	4(16.7)	2(20)	1(25)	27(43.5)	
Gentamicin(10µg)	S	N (%)	12(50)	24(100)	8(80)	4(100)	48(77.4)	
	R	N (%)	12(50)		2(20)		14(22.6)	<0.001
Imipenem(10µg)	S	N (%)	24(100)	24(100)	10(100)	4(100)	62(100)	NS
	I	N (%)	0	0	0	0	0	
Kanamycin(30µg)	S	N (%)	4(16.7)	12(50)	8(80)	2(50)	26(41.9)	
	I	N (%)	4(16.7)	8(33.3)			12(19.4)	<0.01
	R	N (%)	16(66.7)	4(16.7)	2(20)	2(50)	24(38.7)	

P=p- value NS-no statistical significance

Citrobacter spp. isolates from hospital wastewater were 100% resistant to Ampicillinfollowed by Ceftazidim(83.3%), Ceftriaxone (83.3%),Cefotaxim (66.7%) and Gentamicin (33.3%). On the other hand the isolates showed relatively lower resistance against Kanamycin (33.3%), Chloramphenicol and Ciprofloxacin (each 20.8%), No isolate was resistant too Imipenem. Isolates from RDDMH showed 50% resistance rates against: Chloramphenicol, Ciprofloxacin, Gentamicin and Kanamycin. The difference between isolates from RDDMH and hose from other hospitals was statistically significant ($p<0.01$).Isolates from ZMH showed 100% resistance rates against Cefotaxim, Ceftazidim and Ceftriaxone. (Table 9)

Table 9. Drug susceptibility patterns of ***Citrobacter spp.*** isolates from hospital wastewater

Antibiotics	Susceptibility pattern	Sample source				Total count (%)	P
		RDDMH	M ПRH	TASH	ZMH		
Ampicillin(10µg)	S	0	0		0	0	
	R N (%)	8(100)	12(100)	-	4(100)	24(100)	NS
Cefotaxim(30µg)	S N (%)	0	4(33.3)		0	4(16.7)	
	I N (%)	4(50)	0		0	4(16.7)	<0.0001
	R N (%)	4(50)	8(66.7)	-	4(100)	16(66.7)	
Ceftazidim(30µg)	I N (%)	4(50)	0			4(16.7)	<0.0001
	R N (%)	4(50)	12(100)	-	4(100)	20(83.3)	
Ceftriaxone(30µg)	S N (%)		4(33.3)	-		4(16.7)	<0.0001
	R N (%)	8(100)	8(66.7)		4(100)	20(83.3)	
Chloramphenicol(30µg)	S N (%)	4(50)	12(100)	-	3(75)	19(79.2)	<0.001
	R N (%)	4(50)	0		1(25)	5(20.8)	
Ciprofloxacin(5µg)	S N (%)	4(50)	8(66.7)	-	3(75)	15(62.5)	
	I N (%)	0	4(33.3)		0	4(16.7)	<0.001
	R N (%)	4(50)	0		1(25)	5(20.8)	
Gentamicin10µg)	S N (%)	4(50)	12(100)	-	3(75)	19(79.2)	<0.001
	R N (%)	4(50)			1(25)	5(20.8)	
Imipenem(10µg)	S N (%)	8(100)	12(100)	-	4(100)	24(100)	NS
	R N (%)	0	0		0	0	
Kanamycin(30µg)	S N (%)	4(50)	8(66.7)	-	4(100)	16(66.7)	<0.01
	R N (%)	4(50)	4(33.3)			8(33.3)	

P=p-value NS-no statistical significance

All the isolates of *S. aureus* were resistant to ampicillin and amoxicillin (100%). At least one isolate of *S. aureus* was resistant to 7 of the 8 antimicrobials tested. *S.aureus* isolated from the four hospitals showed relatively closer resistance rates against antibiotics tested, so there was no statistically significant difference between hospitals; except, isolates from MΠRH against Erythromycin and those isolated from both MΠRH and ZMH against Cefotaxim showed statistically significant difference ($p<0.01$) than isolates from other remaining hospitals.(Table 10)

Table 10. Drug susceptibility patterns of *S.aureus* isolates from hospital wastewater

Antibiotics	Susceptibility pattern	Sample source				Total count (%)	P
		RDDMH	MΠRH	TASH	ZMH		
Amoxicillin(25µg)	S R N (%)	0 9(100)	0 6(100)	0 6(100)	0 4(100)	0 25(100)	NS
Ampicillin(10µg)	S R N (%)	0 9(100)	0 6(100)	0 6(100)	0 4(100)	0 25(100)	NS
Cefotaxim(30µg)	S N (%) R N (%)	6(66.7) 3(33.3)	3(50) 3(50)	4(66.7) 2(33.3)	2(50) 2(50)	15(60) 10(40)	<0.01
Ceftriaxone(30µg)	S N (%) R N (%)	7(77.8) 2(22.2)	5(83.3) 1(16.)	5(83.3) 1(16.7)	3(75) 1(25)	20(80) 5(20)	NS
Chloramphenicol(30µg)	S N (%) R N (%)	7(83.3) 2(22.2)	4(66.7) 2(33.3)	4(66.7) 2(33.3)	3(75) 1(25)	18(72) 7(28)	NS
Ciprofloxacin(5µg)	S N (%) R N (%)	7(77.8) 2(22.2)	5(83.3) (16.7)	5(83.3) 1(16.7)	4(100) -	21(84) 4(16)	NS
Erythromycin(15µg)	S N (%) R N (%)	8(88.9) 1(11.1)	4(66.7) 2(33.3)	5(83.3) 1(16.7)	3(75) 1(25)	20(80) 5(20)	<0.01
Gentamicin(30µg)	S N (%) R N (%)	8(88.8) 1(11.1)	5(83.3) (16.7)	5(83.3) 1(16.7)	4(100) -	22(88) 3(12)	NS

P= p-value

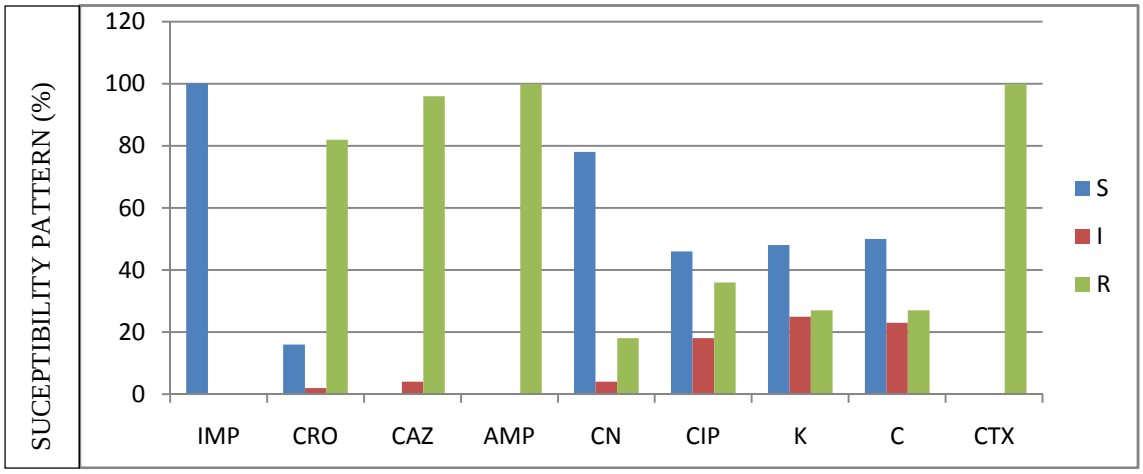
NS-no statistical significance

5.3. Antimicrobial susceptibility pattern in bacterial isolates from hospital vs. non-hospital samples

Although the types of gram negative bacteria isolated from hospital and non-hospital environments were similar, their antimicrobial resistance patterns were different as shown in the figures below

For *Pseudomonas spp.* (Fig 3 below), 100% resistance was observed for Cefotaxim and Ampicillin for both Hospital and Non-Hospital isolates. Highest sensitivity was observed in Imipenem (100%)for both environments. However, a statically significant difference ($p<0.0001$ for all drugs) in resistance to Ceftriaxone (82% vs. 62%), Ceftazidim (96% vs. 62%), Gentamicin (18% vs. 0%), Ciprofloxacin (36% vs. 4%), Kanamycin (27% vs. 4%) and Chloramphenicol (27 vs. 0%)was observed between hospital and non-hospital isolates, respectively.

Panel A



Panel B

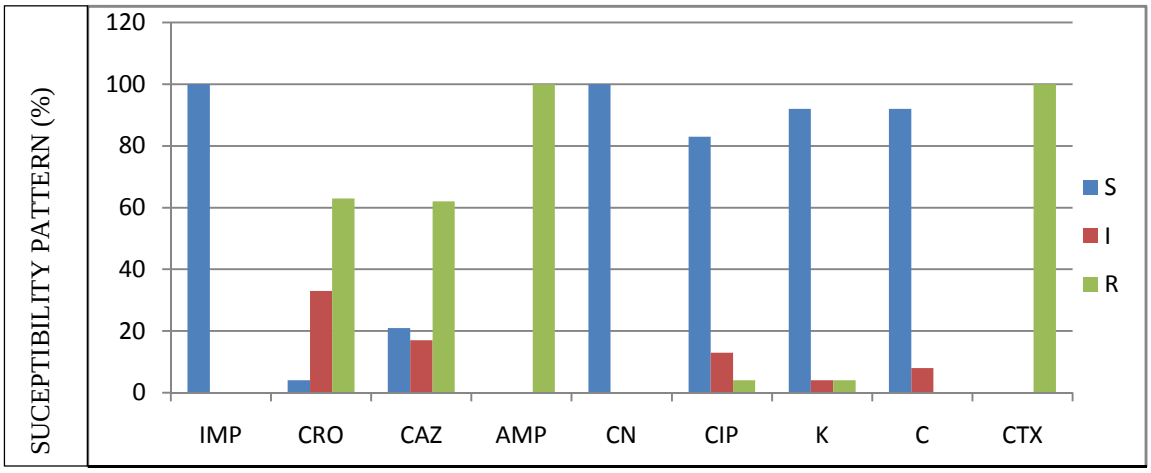
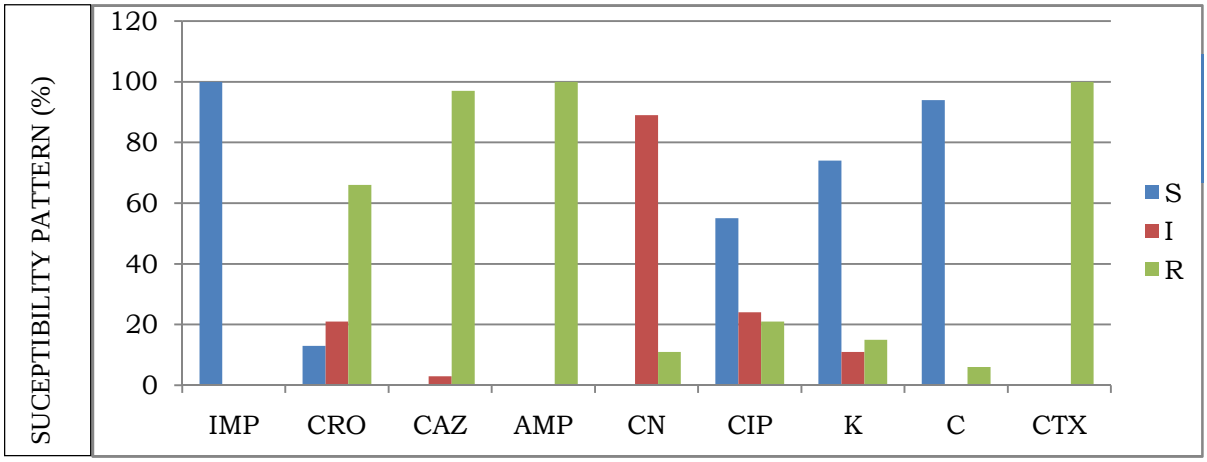


Figure 5. Drug resistance patterns of ***Pseudomonas spp.*** isolated from hospital (panel A) and non-hospital (Panel B) wastewaters Addis Ababa,2016/17

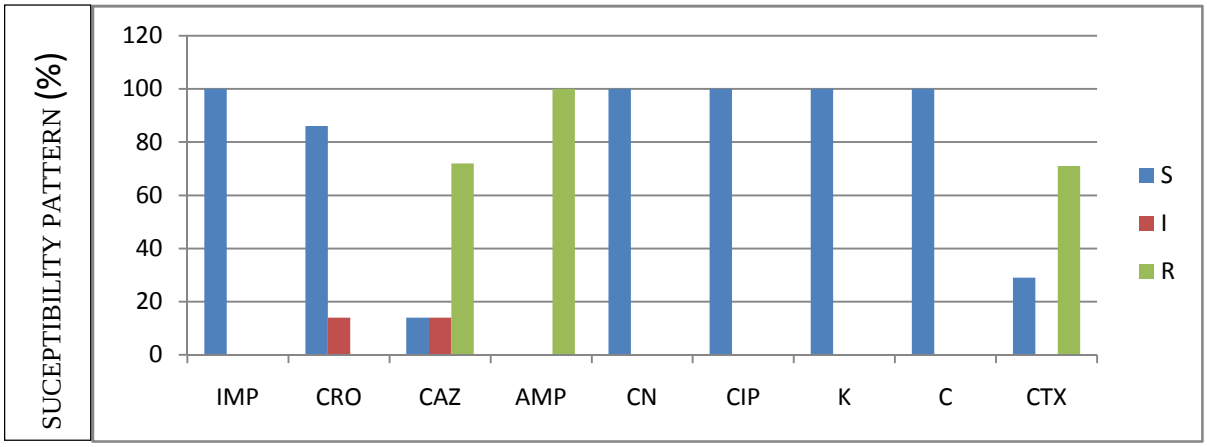
KEY:- AMP-Ampicillin C- Chloramphenicol CIP- Ciprofloxacin CRO-Ceftriaxone IMP- Imipenem CN- Gentamicin CAZ- Ceftazidim CTX-Cefotaxim K- Kanamycin

For *Klebsiella spp.* (Fig 4) lower resistance was observed in both environments against Chloramphenicol, Ciprofloxacin, Kanamycin and Gentamicin with the following rates;(6% vs. 0%), (36% vs. 0%), (15% vs. 0%), (11% vs. 0%) for Hospital and Non-Hospital isolates respectively. Higher resistance was observed against Ceftriaxone (66% vs. O %), Ceftazidim (97% vs. 72%), Cefotaxim (100% vs. 71%) and Ampicillin (100% vs. 100%) among Hospital vs. Non-Hospital isolates respectively. Statistically significant difference ($p<0.001$) was documented in antimicrobial resistance rate to Ceftriaxone (66% vs. O %), Ceftazidim (97% vs. 72%), Gentamicin (11% vs. 0%), Ciprofloxacin (21% vs. 0%), Kanamycin (15% vs. 0%) and Cefotaxim (100% vs. 71%) between hospital and non-hospital isolates, respectively.

PanelA



Panel B



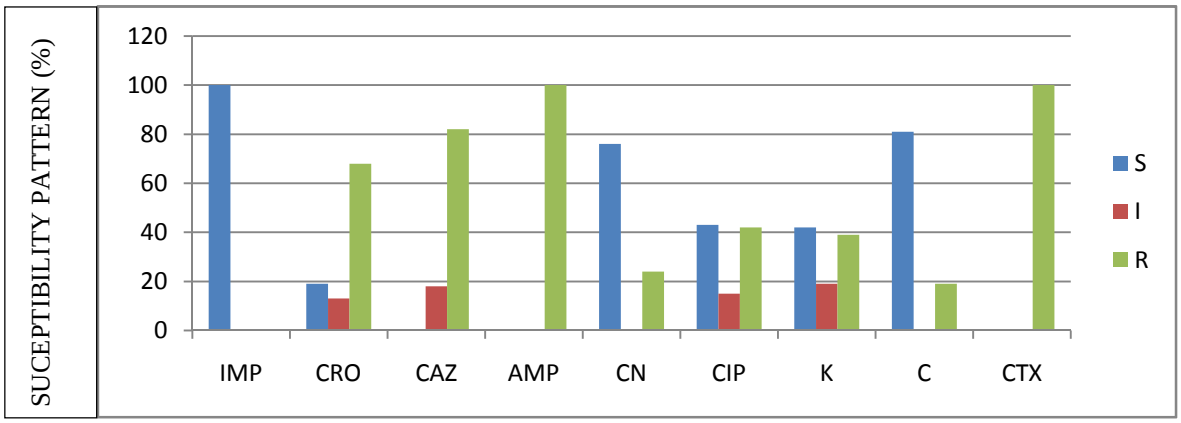
Tested drugs

Figure 6. Drug resistance patterns of ***Klebsiella spp***. isolated from hospital(Panel A) and non-hospital(Panel B) wastewaters Addis Ababa,2016/17

KEY:- AMP-Ampicillin C- Chloramphenicol CIP- Ciprofloxacin CRO-Ceftriaxone IMP- Imipenem CN- Gentamicin CAZ- Ceftazidim CTX-Cefotaxim K- Kanamycin

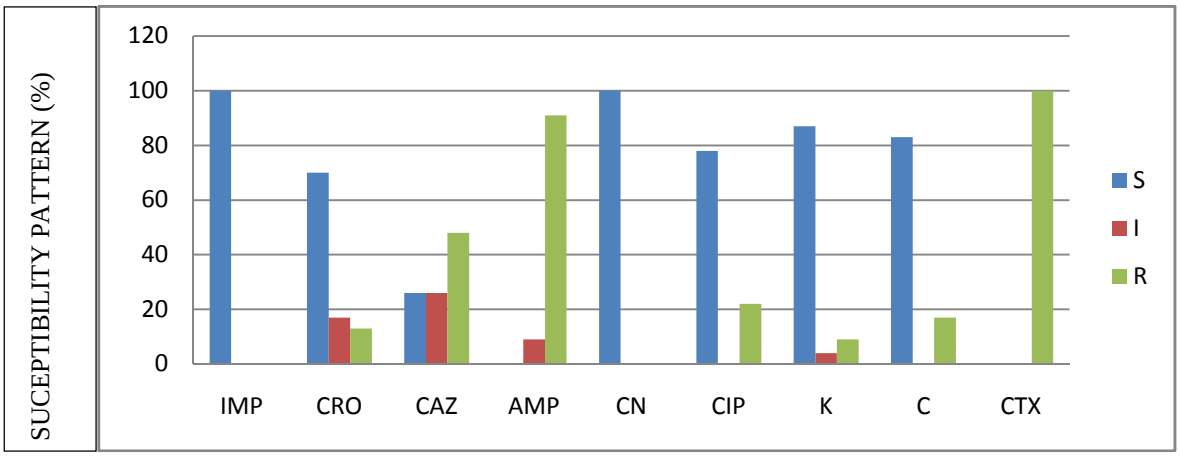
All the isolates of *E.coli* (Fig 5) from both Hospital and Non-Hospital environments were resistant to Cefotaxim (100%). At least one isolate of *E.coli* from hospital environment was resistant to six of the nine antimicrobials tested including Gentamycin. However, no isolate of *E.coli* isolated from non-hospital environment was resistant to Gentamycin in addition to Imipenem, and Chloramphenicol. Hospital isolates were also 100% sensitive to Imipenem. Resistance rate difference to Ceftriaxone (68% vs. 13%), Ceftazidim (82% vs. 48%), Gentamicin (24% vs. 0%), Ciprofloxacin (42% vs. 22%) and Kanamycin (39% vs. 9%); were statistically significant ($p<0.0001$) between hospital and non-hospital isolates, respectively.

Panel A



Tested drugs

Panel B



Tested drugs

Figure 7. Drug resistance patterns of ***E.Coli spp***. isolated from hospital (Panel A) and non-hospital(Panel B)wastewaters Addis Ababa,2016/17

KEY:- AMP-ampicillin C- Chloramphenicol CIP- Ciprofloxacin CRO-Ceftriaxone IMP- ImipenemCN- Gentamicin CAZ- CeftazidimCTX-Cefotaxim K- Kanamycin

Citrobacter isolates (Fig 6) from Non-Hospital environment were 100% sensitive to Imipenem, Ceftriaxone, Gentamicin, Kanamycin and Chloramphenicol but those from Hospital isolates were 100% sensitive to Imipenem only. Non-Hospital isolates showed slightly higher resistance against Cefotaxim (67%) than hospital isolates (62%). There was statistically significant difference between hospital and non-hospital isolates ($p<0.0001$) in resistance to antimicrobial susceptibility testing to Ceftriaxone (83% vs. 0%),Ceftazidim(100% vs. 83%), Gentamicin (21% vs. 0%), Ciprofloxacin (63% vs. 0%), Kanamycin (33% vs. 0) and Chloramphenicol (21% vs. 0%), respectively.

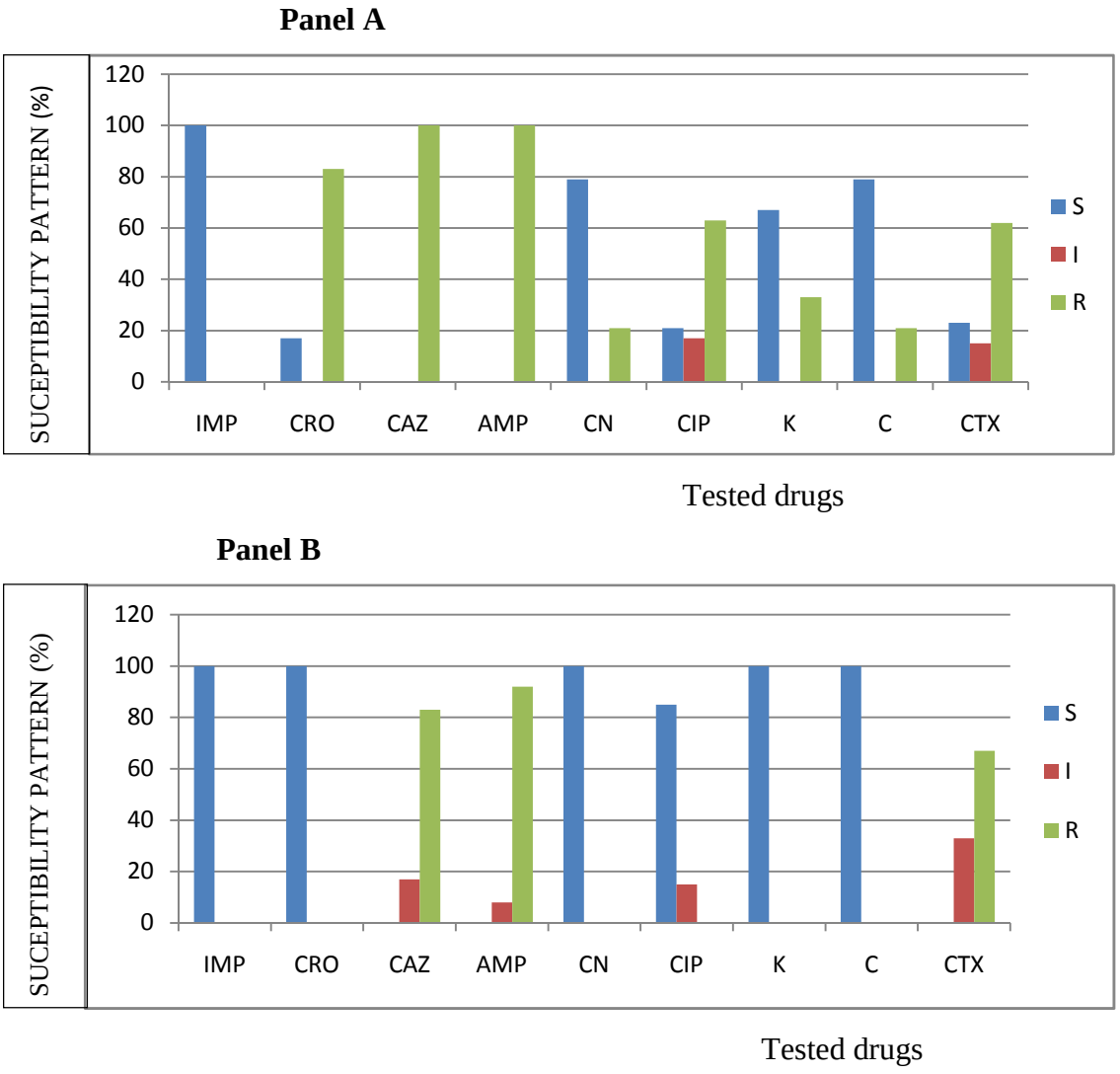


Figure 8. Drug resistance patterns of ***Citrobacter spp***. isolated from hospital(Panel A) and non-hospital (Panel B) wastewaters Addis Ababa,2016/17

KEY:- AMP-Ampicillin C- Chloramphenicol CIP- Ciprofloxacin CRO-Ceftriaxone IMP- Imipenem CN- Gentamicin CAZ- Ceftazidim CTX-Cefotaxim K- Kanamycin

For practical purpose, all Intermediate resistance level reported here for *S. aureus* was considered as resistance. While all the *S. aureus*(100%)isolates from both hospital non-hospital wastewaters were resistant to Ampicillin, resistance against Amoxicillin was 100% for hospital but 83% for non-hospital isolates. However, no isolates of *S. aureus* isolated from non-hospital environment were resistant to Gentamycin and Erythromycin; whereas 40% and 20%, respectively, of those from hospital showed resistance to these drugs. Regarding resistance of *S. aureus* to the other antimicrobials a significant difference ($p<0.01$)was observed between hospital and non-hospital environment as follows: resistance to Ceftriaxone (20% vs. 8%), Amoxicillin (100% vs. 83%), Gentamicin (12% vs. 0%), Cefotaxim (40% vs. 25%), Erythromycin (20% vs. 0%) and Chloramphenicol (28 vs. 17%) for hospital and non-hospital isolates, respectively. (Figure 7)

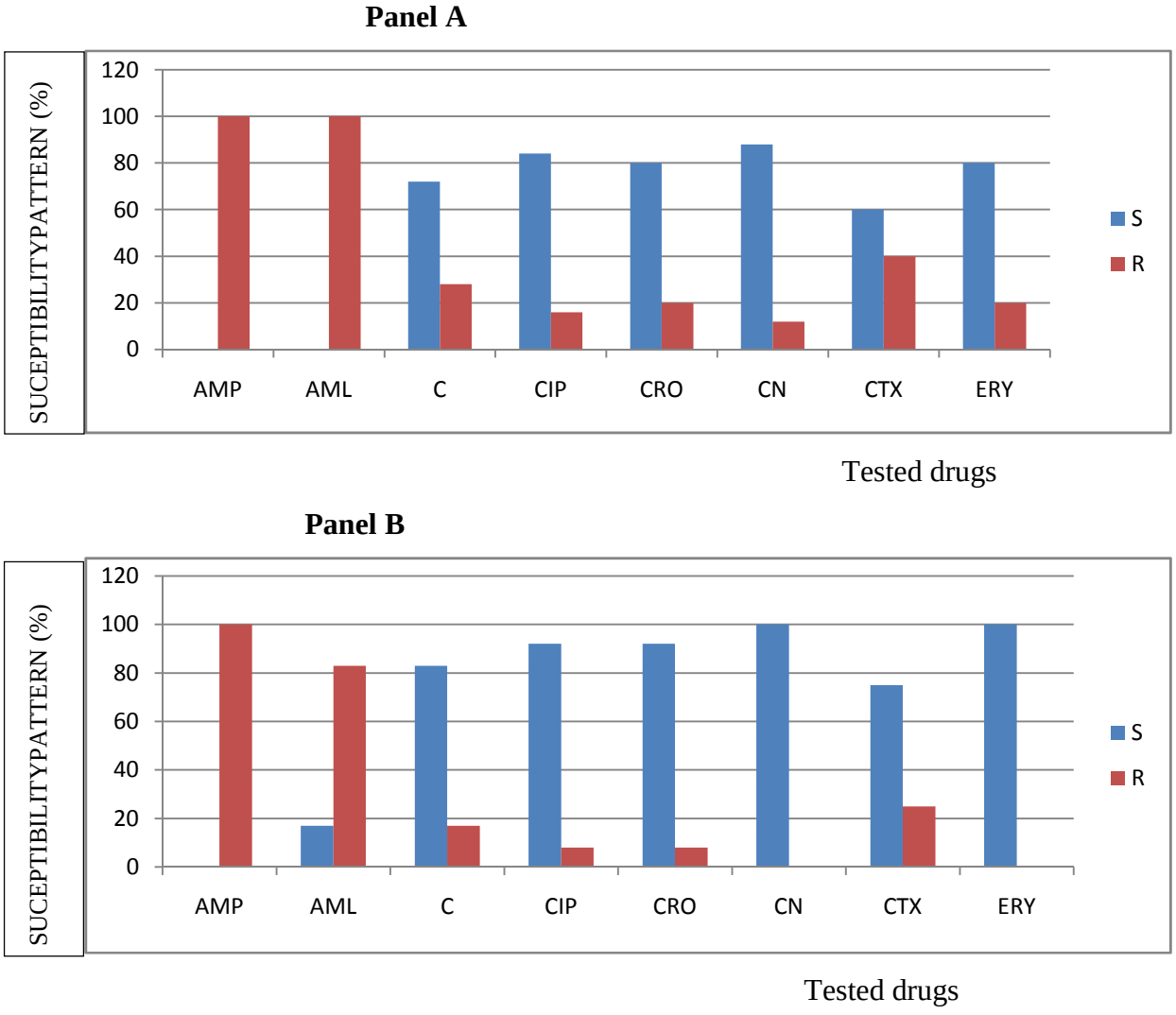


Figure 9. Drug resistance patterns of *Staphylococcus aureus* isolated from hospital(Panel A) and non-hospital (Panel B)wastewaters in Addis Ababa,2016/17

KEY:- AMP-Ampicillin AML-Amoxicillin C-Chloramphenicol CIP-Ciprofloxacin CRO-Ceftriaxone CN-Gentamicin CTX-Cefotaxime ERY-Erythromycin

The overall resistance of gram-negative bacteria isolated from hospital and non-hospital sewage to commonly used antibiotics in the study area was follows: Ampicillin (100% vs. 95.5%), (Imipenem 0% vs. 0%) and Cefotaxim (96.9% vs. 89.6%) with no statistically significant difference ($p>0.05$). However, there was statistically significant difference ($P<0.001$) between hospital and non-hospital wastewater for the rest of antibiotics tested as shown; Ceftriaxone (75% vs. 26.9%),Ceftazidim (91.9% vs. 65.7%), Gentamicin (17.7% vs. 4.5%), Ciprofloxacin (36.2% vs. 8.9%), Kanamycin (27.3% vs. 4.5%) and Chloramphenicol (19.6% vs. 6%).(Table 11)

Table 11. Comparison of overall antimicrobial resistance of gram- negative bacteria between Hospital and non- hospital sewage isolates in Addis Ababa,2016/17

Antibiotics/concentration* in (μg)	Hospital isolates (n=280)(%)	non-hospital isolates (n=156)(%)	p**
Ampicillin(10)	100	95.5	NS
Chloramphenicol(30)	19.6	6	< 0.001
Ceftazidim(30)	91.9	65.7	< 0.001
Kanamycin(30)	27.3	4.5	< 0.001
Ceftriaxone(30)	75	26.9	< 0.001
Ciprofloxacin(5)	36.2	8.9	< 0.001
Imipenem(10)	0	0	NS
Gentamicin(10)	17.7	4.5	<0.001
Cefotaxim(30)	96.9	89.6	NS

**P< 0.001 (p-value)=There is statistically significant difference

NS- no statistical significance

6. Discussion

From the total of 220 hospital and non-hospital sewage samples that were collected from 4 selected hospitals and 4 non-hospital sampling sites, 506 pathogenic and potentially pathogenic bacterial strains were isolated. Among these isolates, 327 (64.6%) were from hospital environment and 179 (35.37%) from non-hospital environments. The rate of isolation of bacterial pathogens in the hospital environment was significantly higher than that in the non-hospital environment (P = 0.001). The most frequently identified bacterium was *Pseudomonas spp*., 160 (31.8%) followed by *E.coli*, 108(21.5%);*Klebsiella spp*.,76 (15%);*Citrobacter spp*. 50(10%);*S. aureus* 37(7.4%) and *Enterobacter spp.*,14(2.8%). Similar trends were reported by (Elmanama *et al*., 2006) in a study conducted to compare the resistance profile of bacterial isolates from Al-Shifa hospital in Gaza and that of a non-health institution, in which the leading isolate was *Pseudomonas spp.*with 33.1% resistance rate *followed by E. coli*, 30.5%; , *Klebsiella spp*., 10.4%;*Proteus spp*., 4.5%; and *Enterococcus spp.,* 21.4%. However, similar study on hospital wastewater in Nigeria showed a bitdifferent rate of isolation for each of the above bacteria: *Pseudomonas* spp. 17 (27.9%) followed by *E. coli* 16(26.2%), *Staphylococcus aureus* 15(24.6%) and *Salmonella* spp. 13(21.3%) (Onuoha, 2017).In a setting similar to that of the present study, Moges*et al*.(2014) in North West Ethiopia identified the same bacterial isolates with different proportion from waste water in hospital and non- hospital environments:: *Klebsiella spp.*30 (26.6%) followed by *Pseudomonas spp.* 19(16.8%), *E. coli* (11.5%) and *Citrobacter spp*. (11.5%), and *Staphylococcus aureus* (8.2%).

Even though, the rate of isolation of bacterial pathogens in the hospital environment was significantly higher than that in the non-hospital environment, qualitatively, the bacterial populations of the hospital and non-hospital isolates were similar in this study; except that no *Proteus* was isolated from non-hospital environment(Table 3).Similar findings were reported by Moges *et al*.(2014) and Elmanama *et al*. (2006), who showed that types of bacteria isolated from hospital and non-hospital wastewater were similar with slight differences. Also wastewater samples were collected from different places with in the same hospital (influent, septic tanker and effluent); there were some notable differences in number of isolates between places in RDDMH isolates. Some isolates were found in the influent samples only, but some were in the septic tank or effluent samples; this may be

because of wastewater inter in to the sewerage system from other sources that where we didn't took sample. Direct comparisons of these findings to previous researches were difficult mainly due to difference in type of wastewater sampled sites. In this research each sample from each sapling point of the same sampling site analyzed separately but in the most of the previous researches wastewater samples collected from different points of the sampling site similar to the present study but finally analyzed by mixing together unlike in present study.

Antibiotic susceptibility test results in this study showed that there were higher resistance rates among bacterial isolates from the hospital sewage to almost all the tested antimicrobial agents compared to those in the non-hospital sewage isolates except for Cefotaxim among *Citrobacter spp*., where it was 67% in non-hospital but 62% in hospital wastewater. Particularly, the gram-negative bacteria isolates from the hospital sewage had significantly ($P < 0.001$) higher resistance rates for six of the nine tested antimicrobial agents as compared to non-hospital sewage isolates (Table 11). The higher multi drug resistance rate from the hospital sewage suggests that the isolated bacteria have been well exposed to antibiotics since continual exposure of bacteria to antibiotics selectively eliminates susceptible ones offering the resistant ones a conducive environment for their increased chance of survival and expansion (Adam, 2016; Bhattacharjee, 2016).

Among both the hospital and non-hospital sewage isolates of gram negative bacteria in this study, the highest resistance was shown against Ampicillin (100% and 95.5%) followed by Ceftazidim (91.9% and 65.7%), Ceftriaxone (75% and 26.9%) and Cefotaxim (96.9% and 89.6%), while the antibiotics with the highest activity were Imipenem (with resistance rate of 0.0% and 0.0%) Gentamycin (with resistance rate of 17.7%and 4.5%) and Chloramphenicol (with resistance rate of 19.6% and 6%), respectively. This is in concordance with previous findings in which low resistance was reported to Imipenem (0.0%) (Elmanama *et al*. 2006), Chloramphenicol and Gentamycin (18% and 14%) (Moges *et al*., 2014). The latter investigators also reported high resistance against Ampicillin (97%). However, the high resistance rate for Ceftazidim, Ceftriaxone and Cefotaxim (91.9%, 75% and 96.9%, respectively) observed in this study is in contrast to what was reported by Moges *et al.* (2014) for Ceftriaxone (20%) and Cefotaxim (33%);Elmanama *et al.* (2006) for Ceftazidim (8.3%); and Cefotaxim (14%) and Rabbani

et al.-(2012) for Ceftazidim (40%) and Cefotaxim (45%). This difference is may be due to the extensive use of these drugs in the study area could have contributed to large-scale dissemination of multi-resistant pathogens in the hospital environment. This idea was supported by Elizabeth *et al*. (2013);that the most important emerging public health threats is that of large-scale dissemination of multi-resistant pathogens in the hospital environment, the community, and the wider environment. Rapid demographic, environmental, and agricultural changes are all contributing to a global antibiotic resistance crisis. Many antibiotics are not inherently biodegradable and some synthetic antibiotics can persist in soils for long periods of time at high concentrations (Elizabeth *et al*. 2013). A range of antibiotics have been detected in soils, surface water, sediments, and groundwater, including fluoroquinolones, sulphonamides, tetracyclines, and macrolides. Although the reported concentrations of antibiotics are generally low (e.g.<1 mg/L in surface waters), the substances have been recorded throughout the year across various hydrological, climatic, and land-use settings. Some substances (e.g. the tetracyclines and fluoroquinolones) also persist in the environment for months to years (Elizabeth *et al*. 2013).

Multiple drug resistance was also common among gram positive isolates to commonly used antibiotics in the tested samples. Antibiotic susceptibility pattern of Staphylococcus was well studied in clinical sample; however very limited report is available on resistance pattern of isolates from hospital sewage sample. All isolates of *S. aureus* from this study were resistant to Ampicillin (100%) and Amoxicillin (100%). Relatively reduced resistance was observed against: - Chloramphenicol (28%), Ciprofloxacin (20%), Gentamycin (12%), Cefotaxim (40%), Erythromycin (20%) and Ceftriaxone (20%). Similar findings were reported by Moges *et al.*(2014) and Fekadu *et al.*(2015) with some notable differences. *Staphylococcus aureus* were detected in high number in all sewage samples from both hospital and non-hospital environments, indicating their continuous release to the receiving environment. This is in line with other studies which reported that the organism is resistant to antiseptics, disinfectant and several antibiotics and survives in the sewerage system for long period (Nunez and Moretton, 2007;Ekhaise and Omavwoya, 2008;Aparecida*et al*, 2000). Contamination of river and lake with this pathogen may pose risk to the public health associated with *Staphylococcal* infection and food poisoning

The overall prevalence of multiple drug resistance (MDR)(resistance to two or more drugs) in this study was 392/506 (77.5%).Multiple drug resistance in hospital environment was found to be280 /327 (85.6%), while in non-hospital environment it was 112/179 (62.6%), which was statistically significant (p=0.01). When we consider isolates showing resistance to more than 5 antibiotics, the distribution of MDR isolates in hospital environment was still found to be significantly higher (P=0.001)than non-hospital environments with the rate of 183/327 (56%) and 28/179 (15.6%), respectively. Similar findings from elsewhere in the country showed that multiple drug resistance against 2–12 antibiotics was found to be 79/113 (69.9%)(Moges *et al.*, 2014). The same authors reported that multi-drug resistance in hospital environment was 53/65 (81.5%) while in non-hospital environment was 26/48 (54.2%).

Limitations

- ✓ Although *Enterococcus* is important gram positive bacteria in wastewater, this study failed to isolate and determine its drug resistance pattern mainly due to lack of selective bacteriologic media.
- ✓ Current study was conducted for only five weeks which cannot be representative for effluent discharges for the duration of all seasons in a year; therefore, findings of this study may not indicate actual situation in the study area throughout a year.
- ✓ In this study, isolated gram negative bacterial strains were not typed mainly due to lack of biochemical test and serological kits.
- ✓ Another limitation of this study was failure to select all antimicrobial agents commonly used for resistance evaluation. Those antimicrobial agents not included into this study were either due to not being used for routine susceptibility tests or not available during the study period because of antibiotics cycling policy in these hospitals.
- ✓ This study was done in selected hospitals only. A large scale study is needed for definite conclusions**.**

Conclusions

High numbers of bacteria were isolated from both hospital and non-hospital wastewater samples. This is an indication for possible presence of pathogenic organisms that are discharged into receiving environment (lake and river) posing risk to public health. The absence of hospital sewage treatment and low habit of disinfection before releasing waste water in to sewerage system may contribute to the dissemination of such multi-drug resistant bacteria from the hospital to the environment by draining those bacteria into the city sewage pool or directly into the water bodies such as lakes and rivers. The contamination of hospital sewage by antibiotics or other pollutants leads to the rise of resistance due to selection pressure. This study showed that the hospital sewage isolates had significantly higher antibiotic resistance rates than the non-hospital isolates.

Recommendations

- Hospital wastewater should be treated by appropriate wastewater treatment plant before released into the environment to minimize dissemination of pathogenic and potentially pathogenic bacteria to receiving environment.
- As hospital effluents carry chemical and microbiological hazard, quality of effluent discharged in to receiving environment (water bodies) should be assessed on a regular basis to minimize risk to public health.
- Antibiotic susceptibility pattern of pathogenic and potentially pathogenic bacteria in hospital effluent should also be regularly tested to determine the magnitude of environmental pollutions due to dissemination of antimicrobial resistant organisms so that concerned policy makers would pay due attention to formulate a strategy in the right direction.
- Large scale studies that also includes drug resistance gene assessment should be conducted in the country to reveal healthcare liquid waste management and microbiological quality of effluents discharged into receiving environment

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Appendixes

1. **Laboratory procedures**

1.1. Detection and isolation of pathogenic & potentially pathogenic bacteria

1. *Pseudomonas spp*.

Procedure

1. Inoculate 0.5ml of ten-fold diluted sample on pseudomonas agar plate
2. Incubate inoculated plates aerobically at 35-37ºc
3. Examine inoculated plates after 24 - 48 hours.
4. The presence of blue-green or brown pigmentation may be considered as presumptive evidence of *Pseudomonas aeruginosa*.

2. *E. coli* (potentially pathogenic)

Procedure

1. Inoculate 0.5ml of ten-fold diluted sample on MacConkey agar plate
2. Incubate inoculated plates aerobically at 35-37ºc for 24houres
3. Pick smooth pink colonies and sub-culture on MacConkey agar plate
4. After getting pure colonies perform biochemical tests

3. *Klebsiella spp.*

Procedure

1. Inoculate 0.5ml of ten-fold diluted sample on MacConkey agar plate
2. Incubate inoculated plates aerobically at 35-37ºcfor 24h.
3. Pick mucoid pink colonies an sub –culture on MacConkey agar
4. Perform biochemical test by taking pure colonies

4. *Citrobacter spp.*

Procedure

1. Inoculate 0.5ml of ten-fold diluted sample on MacConkey agar plate
2. Incubate aerobically at 35-37ºc for 24 hours.
3. Pick smooth pale to pink coloured colonies and sub-culture on MacConkey agar
4. Perform biochemical tests from pure colonies

5. Staphylococcus aureus

Procedure

1. Inoculate ten- fold diluted sample on manitol salt agar (MSA)
2. Incubate at 35-37ºc for 24-48 hours
3. Pick manitol fermenting yellow colonies & subculture on nutrient agar
4. Take colonies from nutrient agar and Perform Gram reaction (staphylococcus species are gram positive with grape morphology)
5. Perform slide catalase test using 3 % hydrogen peroxide (staphylococcus species are catalase positive)
6. Slide Coagulase test identify *S. aureus* from other species

1.2.Gram staining technique

1. Make smear from colonies grown on basic media
2. Fix the dried smear
3. Cover the fixed smear with crystal violet stain for 30–60 seconds.
4. Rapidly wash off the stain with clean water.
5. Tip off all the water, and cover the smear with Lugol's iodine for 30–60 seconds.
6. Wash off the iodine with clean water.
7. Decolorize rapidly (few seconds) with acetone–alcohol.
8. Wash immediately with clean water.

Caution: Acetone–alcohol is highly flammable; therefore use it well away from an open flame.

9. Cover the smear with neutral red/sufranin stain for 1 minute.
10. Wash off the stain with clean water.
11. Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry.
12. Examine the smear microscopically, first with the 40X objective to check the staining and to see the distribution of material, and then with the oil immersion objective to report the bacteria and cells.

1.3 Antibiotic susceptibility testing

Method: disk diffusion

1. Using a sterile wire loop, touch 3–5 well-isolated pure colonies of similar appearance to the test organism and emulsify in 3–4 ml of sterile physiological saline.
2. In a good light match the turbidity of the suspension to the turbidity standard (mix the standard immediately before use). When comparing turbidities it is easier to view against a printed card or sheet of paper.
3. Using a sterile swab, inoculate a plate of Mueller Hinton agar. Remove excess fluid by pressing and rotating the swab against the side of the tube above the level of the suspension Streak the swab evenly over the surface of the medium in three directions, rotating the plate approximately 60º to ensure even distribution.
4. With the petri dish lid in place, allow 3–5 minutes (*no longer than 15 minutes*) for the surface of the agar to dry.
5. Using sterile forceps, needle mounted in a holder, or a multidisc dispenser, place the appropriate antimicrobial discs, evenly distributed on the inoculated plate.

Note: The discs should be about 15 mm from the edge of the plate and no closer than about 25 mm from disc to disc. No more than 6 discs should be applied (90 mm dish). Each disc should be lightly pressed down to ensure its contact with the agar. It should not be moved once in place.

6. Within 30 minutes of applying the discs, invert the plate and incubate it aerobically at 35ºC for 16–18 h.
7. After overnight incubation, examine the control and test plates to ensure the growth is confluent or near confluent. Using a ruler on the underside of the plate measure the

diameter of each zone of inhibition in mm. The endpoint of inhibition is where growth starts.

8. Zone diameters are converted into different susceptibility categories using the zone/MIC interpretive criteria from the most recent annually published CLSI M100 series documents for disk diffusion using the appropriate tables for the organism being tested. Organisms are categorized as susceptible, intermediate, or resistant to the antibiotics tested.

Declaration

I hereby declare, in accordance with the by-laws of the University of Addis Ababa, the thesis work described herein is entirely own work of Redwan Mohammed. This thesis contains no material previously published or written by another person, except where duly referenced. he work embodied in this project, is original and has not been submitted in part or full for any degree of this University or any other institutions.

……. ……

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