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ASSESSMENT OF DISINFECTANT AND ANTIBIOTIC RESISTANT BACTERIA IN HOSPITAL WASTEWATER, SOUTH ETHIOPIA

BY

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Operational Definitions

Antibiotics**:** Chemical agents obtained from living microorganisms and able to kill or inhibit growth of other microorganisms

Composite sample: Partial samples collected at varies times of the day and finally mixed up in larger container

Disinfectants: Chemicals that are applied on inanimate objects to reduce microbial load

Disk diffusion: Method of antibiotic susceptibility testing using disks impregnated with known concentration of antibiotic

Effluent: Wastewater discharged into receiving environment

Geometric mean: Parameter used to calculate bacterial population

Hospital wastewater: Liquid waste generated in varies section of the hospital

Indicator organisms: Group of bacteria that are used to assess bacteriological quality of water and wastewater

Influent: Wastewater entering to treatment plant

Most probable number: Method of bacterial enumeration in water and wastewater using series of tubes containing growth medium

Reduction factor: Number of log reduced bacteria after wastewater treatment with disinfectant solution

$$[\text{Reduction factor} = \log_{10} \text{CFU/mL}_{\text{(negative control)}} - \log_{10} \text{CFU/mL}_{\text{(treated)}}]$$

Resistance: Bacteria unable to respond for a given antibiotic

Susceptible**:** Bacteria able to respond for a given antibiotic

Wastewater treatment: A process that used to reduce chemical and biological hazard from wastewater

Acronyms

AAU: Addis Ababa University

APHA: American Public Health Association

CFU: Colony Forming Unit

CRAs: Chlorine Releasing Agents

DMIP: Department of Microbiology, Immunology and Parasitology

ECC: Escherichia coli Count

EC-MUG: *Escherichia coli* 4-methylumbelliferyl-β-glucuronide

ENC: Enterococcal count

FCC: Fecal Coliform Count

HPC: Heterotrophic Plate Count

HURH: Hawassa University Referral Hospital

HWW: Hospital Wastewater

MIC: Minimum Inhibitor Concentration

MPN: Most Probable Number

SNNPR: Southern Nation, Nationalities and Peoples' Region

TCC: Total Coliform Count

THC: Total Heterotrophic Count

YAH: Yirg Alem Hospital

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Abstract

Background*: Large quantities of antibiotics and disinfectants are used in hospitals for patient care and disinfection process, respectively. These products are partially metabolized and residual quantities reach hospital wastewater, exposing bacteria to wide range of biocides that could act as selective pressure for development of resistance.*

Objectives*: The aim of the study was to assess disinfectant and antibiotic resistant bacteria in Yirg Alem and Hawassa hospitals effluents.*

Methods*: A cross-sectional study was conducted from December 2010-February 2011 in hospital wastewater. A total of 24 composite samples were collected on weekly basis for bacteriological analysis and susceptibility testing. Indicator organisms, pathogenic and potentially pathogenic bacteria were determined and isolated on selective bacteriologic media. Disinfectant activity was evaluated in use-dilution for tincture iodine, sodium hypochlorite and 70% ethanol and MIC was determined by agar dilution method. Similarly antibiotic susceptibility test were performed using Kirby-Bauer disk diffusion method. All methods were used according to standard methods for examination of water and wastewater.*

Results: *Pathogenic (Salmonella, Shigella and S. aureus) and potentially pathogenic (E. coli) bacteria were detected from effluents of both hospitals. Activity of disinfectant in use-dilution demonstrated tincture-iodine as the most effective agent, followed by sodium hypochlorite and the least active was 70% ethanol. MIC for ethanol against S. aureus and gram negative rods from Yirg Alem hospital (YAH) showed 4 and 3.5 log reduction, respectively. Similarly 3.8 and 3.2 log reduction were observed for S. aureus and gram negative rods from Hawassa University Referral Hospital (HURH), respectively. Salmonella isolates from YAH effluent were resistance to Ceftriaxone, Tetracycline and Doxycycline, whereas from HURH effluent were resistant to the above three antibiotics and Gentamycin too. S. aureus from YAH effluent was resistant to Penicillin, Ampicillin and Amoxicillin, whereas from HURH was resistant to the above three antibiotics and Gentamycin too.*

Conclusion*: Hospital effluents tested contain antibiotic resistant bacteria which are released to receiving water bodies resulting in huge public health threat.*

Key words*: antibiotics, biocides, disinfectants, heterotrophic count, hospital effluent, hospital influent, indicator, minimum inhibitory concentration, most probable number*

Chapter I

Introduction

Background

Wastewater is referred to any water, whose quality has been adversely being abused by anthropogenic influence. This includes liquid waste discharged from domestic home, agricultural commercial sectors, pharmaceutical and hospital. Hospitals are an essential asset of any society, and waste production is inevitable outcome of service delivery. In hospitals water consumed by various parts such as hospitalization, surgery rooms, laboratories, administrative units, laundry, health services, kitchen and in the process its physical, chemical and biological quality decreased and converted to wastewater (Mahvi et al, 2009). Health care waste consists of solid, liquid and gaseous waste contaminated with organic and inorganic substance including pathogenic microorganisms, radiological chemicals, partially metabolized antibiotics which are usually generated from laboratory analysis of tissues and body fluids as well as excreted from patients (Nuñez & Moretton, 2007).

A variety of substances such as pharmaceuticals, radionuclides, antiseptics, disinfectants and solvents are used in hospitals for treatment, medical diagnostics, disinfection and research. After application many non-metabolized drugs excreted from patients and residual chemicals enter into wastewater which finally interacts with microflora of Hospital sewage. These microflora are composed by saprophytic bacteria from the atmosphere, soil, medical devices and water employed in the hospital practice; the pathogens are mainly released with the patient excreta. These bacteria that survive in Hospital wastewaters may be exposed to a wide range of biocides that could act as a selective pressure for the development of resistance. Due to heavy antibiotic use, hospital wastewater contains larger numbers of resistant organisms than domestic wastewater (Abdelraouf et al, 2006). When the antimicrobial agents attack disease-causing bacteria, they also affect non-pathogenic bacteria in their course, thus they exterminate these bacteria and make room for more resistant bacterial growth (Nuñez & Moretton, 2007).

It is clear that microorganisms can adapt to a variety of environmental, physical and chemical conditions, and it is therefore not surprising that resistance to extensively used antibiotic, antiseptics and disinfectants has been reported. Many of these reports of resistance have often paralleled issues including inadequate cleaning, incorrect product use, or ineffective infection control practices, which cannot be underestimated.

Resistance can be either a natural property of an organism (intrinsic) or acquired by mutation or acquisition of plasmids or transposons. The nature of biofilm structure and the physiological attributes of biofilm organisms confer an inherent resistance to antimicrobial agents, whether these antimicrobial agents are antibiotics, disinfectants, or germicides (Rodney and Costerton, 2002). Intrinsic resistance is mostly demonstrated by gram-negative bacteria, bacterial spores and mycobacterium. Acquired, plasmid-mediated resistance commonly exists in both gram-negative and positive bacteria and most widely associated with many antimicrobial agents which are conferred by R-factor (Olowe et al, 2004). In recent years, rotation of disinfectant**s** and antibiotics in hospitals and elsewhere, e.g. in the pharmaceutical, agricultural and food industries, has been advocated to prevent the development of bacterial resistance (Russell, 2002). It has been speculated that low-level resistance may aid in the survival of microorganisms at residual levels of antibiotics, antiseptics and disinfectants; any possible clinical significance of this remains to be tested. With growing concerns about the development of biocide resistance and cross-resistance with antibiotics, it is clear that clinical isolates should be under continual surveillance and possible mechanisms should be investigated (Gerald and Russell, 1999).

The public health impact of release of resistant bacteria to receiving environment can be explained by many ways. First, if the resistant bacteria are carrying transmissible gene, they transfer resistant genes through conjugation or transduction so that infection caused by these bacteria are usually difficult to treat and also decrease antibiotic pool for treatment of bacterial infection. Second, this organism may act as vector or reservoir of resistant genes. Third, there will be increased nosocomial infection. Fourth, if infection occurs, it will increase cost of treatment and hospitalization (Nuñez & Moretton, 2007).

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 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.

In this study, we have selected Yirg Alem hospital and Hawassa university referral hospital. Both hospitals are located in southern Nations, Nationalities and Peoples' Region (SNNPR) and serving more than 1,500,000 people each. The disinfectant and antibiotic resistance patterns of pathogenic bacteria released from effluents of these two hospitals have not been assessed. Therefore this study is designed to assess pattern of antimicrobial resistance for bacteria isolated from hospital effluents against commonly used antimicrobial agents in the hospitals. The findings of this study will indicate selection of proper antimicrobial agent for hospital use and better management of hospital effluents.

Statement of the Problem

Large amounts of antibiotics and disinfectants are used in hospitals for patient treatment, as antiseptic and disinfection of inanimate objects (Nuñez & Moretton, 2007). After use residual quantities of these agents reach 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 (Vishal et al, 2009). Similarly many research conducted in different countries has shown that presence of antibiotic and disinfectant resistant bacteria in hospital effluents. For instance study conducted in Nigeria showed that co-resistance to disinfectants and clinically relevant antibiotics among the organisms reported was an indication of the risks posed by the untreated effluents to public health. It also adds to the increasing evidence about the role of hospital wastewaters as environmental reservoir of multi-drug-resistant bacteria (Adelow et al, 1997). 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 (Yang et al, 2009).

The resistant bacteria evolve and selected by long-term environmental exposure to the low concentrations of antibiotics and disinfectants (Aparecida et al, 2000). Despite the advance in antimicrobial therapy and disinfection process worldwide, the prevalence of infectious disease caused by antimicrobial resistant organisms is increasing. In view of the importance of disinfection in clinical practice and domestic hygiene, and danger of development of resistance by organisms exposed to disinfectant, it will be in the overall interest of all to ensure that only fresh preparations of disinfectants are used routinely and dilution should be restricted to the concentration ranges that have been found definite activity against the organisms (Hani, 2009). Study conducted in hospitals of north Jordan to determine antibacterial efficacy of common chemical agents in cleaning and disinfection showed that ethanol, ethanol plus glycerin and ethanol iodine were found to be the most effective agents against microorganism tested (Hani, 2009).

The public health impact of high prevalence of resistant organisms in the environment should not be underestimated. Study conducted in Germany revealed possible role played by hospital and municipal wastewater systems in the selection of antibiotic-resistant bacteria and indicating possible transfer of resistant genes from wastewater and surface water to the drinking water distribution network (Schwartz, 2003). Similar study conducted in Gaza, demonstrated that bacteria that has been isolated from wastewater samples from Al-Shifa hospital and laboratory building of Islamic University of Gaza contain higher number of antibiotic resistant bacteria than bacteria that isolated from other sites (Abdelraouf et al, 2006). In Ethiopia, most hospitals do not have wastewater treatment plant which worsens the problem. Moreover the untreated wastewater will join rivers, streams or disposed to underground where some of the water will be used for drinking, domestic, 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 (Mesdaghinia et al, 2009).

The aim of this study is to assess disinfectant and antibiotic resistant bacteria in hospital effluents. The effluent bacteria are tested for resistance to commonly used antibiotics and disinfectants in the hospitals.

Significance of the study

Hospitals are an essential asset of any society, and waste production is inevitable outcome of service delivery. Large amount of antibiotics and disinfectant chemicals are daily used for patient care and control of infection in health institutions. Residual quantities of these biocides, some of are partially metabolized antibiotics which are excreted through feces and urine of patient will reach healthcare liquid wastes, which are reservoirs of harmful biological and chemical hazard. The overall effect of biocides to act as selective pressure on hospital microflora and development of disinfectant and antibiotic resistant bacteria. Moreover if the resistant genes are transmissible and these bacteria reach to community bacterial strains, it results wide dissemination of resistant bacteria and their genes in the environment. Infections caused by antibiotic resistant bacteria are usually difficult to treat, prolong hospitalization and increase cost of treatment. 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 in Ethiopia resistance pattern to common disinfectants and antibiotics in Hospital effluents are not determined, therefore this study aimed to assess the resistance pattern and the findings which will be obtained helps to update Hospital effluent management, proper antibiotic usage and disinfectant selection for hospital use.

Chapter II

Literature review

Large quantities of disinfectants and antibiotics are used in hospitals for disinfection process and patient treatment respectively. Most of the antibiotic taken by the patients are partially metabolized and excreted through feces and urine. After use, residual quantities of these products reach the wastewater, exposing the bacteria that survive in hospital wastewaters to a wide range of biocides that could act as a selective pressure for the development of resistance (Nuñez and Moretton, 2007). Increasing attention has been directed recently to the resistance of bacteria to antibiotics and disinfectants. The resistant bacteria isolated were diverse in nature. For example, study conducted in Buenos Aires City hospital, Brazil, the bacterial population resistant to disinfectants was mainly composed by *Enterobacteriaceae, Staphylococcus* spp, and *Bacillus* spp, which are highly associated to nosocomial infections (Nuñez and Moretton, 2007).

Co-resistance to antimicrobial agents among organisms are reported in different studies and an indication of the risks posed by the untreated effluents to public health as well as increasing evidence about the role of hospital wastewaters as environmental reservoir of multi-drug-resistant bacteria. 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 test antimicrobial agents ranging from 0% to 77.8%. Furthermore the report explained, among 25 organisms isolated from hospital A were recognized 16 phenotypic patterns of co-resistance to the test disinfectants and antibiotics; while from hospitals B and C were recognized 13 and 9 patterns, respectively, from among 18 and 14 isolates (Adelow et al, 1997). However in study conducted to compare disinfectant and antibiotic activities, the result showed all strains tested were susceptible to sodium hypochlorite, glutaraldehyde and to the association quaternary ammonium - formaldehyde - ethyl alcohol disinfectants. Further susceptibility of strains to phenol and to one quaternary ammonium compound was variable. Among twenty-one antibiotic-multiresistant strains (methicillin-resistant *Staphylococci*, *Enterococcus* spp, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter cloacae*, *Serratia marcescens* and *Escherichia coli*) eleven (52%) and eight (38%) strains were resistant to the quaternary ammonium and phenol compounds, respectively. Among six isolates that demonstrated susceptibility to antibiotics (*Staphylococci*, *Enterococcus* spp, *P. mirabilis*, *E. cloacae* and *E. coli*) two strains (33%) showed resistance to these disinfectants. This result demonstrated that lack of correlation between antibiotic-susceptibility and susceptibility to disinfectants in hospital strains (Aparecida et al, 2000). However, one study demonstrated cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* showed that exposure of a clinically significant bacterium to the antiseptic triclosan efficiently can select for multi-drug resistant (MDR) derivatives, including high-level resistance to an antipseudomonas drug (Rungtip et al, 2001).

Healthcare liquid waste 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 difficult for antibiotic treatment. Study carried out in Nepal found out that healthcare liquid wastes were loaded with multiple drug resistance bacteria and seemed to pose a huge public health threat in the transfer of such resistance to the bacterial pathogens causing community acquired infections, thereby limiting our antibiotic pool (Sharma et al, 2010). Also study conducted in Sweden demonstrated that high prevalence of Vancomycin Resistant Enterococci (VRE) in Swedish sewage possibly due to antimicrobial drugs or chemicals released into the sewage system may sustain VRE in the system (Aina et al, 2002).

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 in nature. 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. (33.1%) followed by *E. coli* (30.5%), *Enterococcus* sp. (21.4%), *Klebsiella* sp. (10.4%) and *Proteus* sp. (4.5%). 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 contain higher number of antibiotic resistant bacteria than bacteria that isolated from other sites (Abdelraouf, 2006).

Antimicrobial resistance may spread in aquatic environment (drinking and recreational water) and its role is not only as reservoir of clinical resistance genes, but also as a medium for spread and evolution of resistance genes and their vectors (Hilary-Kay, 1993). Therefore the potential for indigenous aquatic organisms to provide the source of new resistance genes and their associated genetic vectors and to function as hosts for the continued evolution of clinically important resistance genes deserves more intense and detailed investigation (Hilary-Kay, 1993). Study conducted in Brazil detected extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* in effluents and sludge of a hospital sewage treatment plant and indicated the hospital wastewater treatment plant did not show a satisfactory efficacy in removing pathogenic micro-organisms, allowing for the dissemination of multiresistant bacteria into the environment (Pardo et al, 2007). However study conducted in U.S wastewater research division, municipal environmental research laboratory, U.S. Environmental Protection Agency observed the effect of UV light disinfection on antibiotic resistant coliforms in wastewater effluents and indicated UV irradiation effectively disinfected the wastewater effluent, the percentage of the total surviving coliform population resistant to Tetracycline or Chloramphenicol was significantly higher than the percentage of the total coliform population resistant to those antibiotics before UV irradiation and the finding was attributed to the mechanism of R-factor mediated resistance to tetracycline (Mark, 1982). The influence of hospital wastewater containing resistant bacteria discharged to the receiving environment should not be underestimated. Public health impact is one of the most serious and 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 fishfarm water generally showed, Oxytetracycline-resistant hospital heterotrophs displayed a higher frequency (84%) of Ampicillin (Amp) tolerance compared to the Oxytetracycline-resistant heterotrophs from the freshwater fishfarm site (22%) (Huys et al, 2001). Similar study conducted in Austria to evaluate antibiotic resistance of *E. coli* in sewage and sludge, the highest resistance rates were found in *E. coli* strains of a sewage treatment plant which treats not only municipal sewage but also sewage from a Hospital (Reinthaler et al, 2003). Other study conducted in Nigeria demonstrated that hospital wastewater was observed to play a significant role in the influence on 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 (Ekhaise and Omavwoya, 2008). Most of the time hospital effluents with other sewage released to water bodies (lakes, rivers or streams) or underground 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 (Mesdaghinia et al, 2009).

Some microorganisms inherently developed resistance to disinfectant and even grow in disinfectant solutions. The problem becomes worst, if these organisms are present in hospital environment and easily transmitted to patients from medical devices and pose significant health problem. For instance study showed that resistant cells of *Pseudomonas aeruginosa* and a waterborne *Pseudomonas* sp.(strain Z-R) were able to multiply in nitrogen-free minimal salts solution containing various concentrations of commercially prepared, ammonium acetate-buffered benzalkonium chloride (CBC), a potent antimicrobial agent (Frank et al, 1969). Moreover the efficacy of disinfectant should be monitored regularly to insure proper activity against major medically important organisms. One study conducted to show the efficacy of variety of disinfectant against *Listeria* spp. in the presence and absence of organic matter revealed that the presence of whole serum and milk (2% fat) further reduced the disinfectant capacities of most of the formulations studied and findings emphasize the need for caution in selecting an appropriate disinfectant for use on contaminated surfaces, particularly in the presence of organic material (Best et al, 1960). For instance study conducted to reveal factors influencing the occurrence of high number of iodine-resistant bacteria in iodinated swimming pool, iodine appeared to be more effective than chlorine against both the standard fecal indicators, coliform bacteria and *enterococci*, and against *staphylococci* derived from the mouth, nose, and skin (Martin et al, 1966) and explained that high iodine concentrations were easily maintained, and that bathing load, sunlight, and presence of organic matter had less of a depleting effect on the iodine concentrations than on the chlorine concentrations (Martin et al, 1966).

Serious problem of release of antibiotic or disinfectant resistant bacteria to the environment is potential of transferring of the resistant gene to community bacteria. Infection caused by resistant bacteria will challenge the antibiotic therapy and increase the cost of treatment and hospitalization. Study conducted in Philadelphia to examine transfer of plasmids pBR322 and pBR325 in wastewater from laboratory strains of *Escherichia coli* to bacteria indigenous to the waste disposal system showed that bacterial strains isolated from raw wastewater or a plasmid-free *E. coli* laboratory strain served as recipients. Transfer of the pBR plasmid into the recipient strain occurred during a 25-h co-incubation in either L broth or sterilized wastewater (Michael et al, 1985). After the co-incubation, recipients exhibited both plasmid-encoded phenotypic characteristics and an altered plasmid profile, as shown by agarose gel electrophoresis of purified plasmid DNA (Michael et al, 1985). However study conducted in France to trace whether antibiotic-resistant *Pseudomonas aeruginosa* isolated from hospitalized patients recovered in the hospital effluents, the result showed genotyping of both clinical and wastewater isolates was determined by using pulsed-field gel electrophoresis (PFGE). There was no common PFGE pattern in antibiotic-resistant *P. aeruginosa* from humans and wastewater therefore antibiotic-resistance profile of wastewater isolates was different from that of clinical isolates (Tumeo et al, 2008).

Continued updating of the susceptibility pattern of recent clinical isolates, isolates from hospital wastewater and follow-up in changes of antibiotic patterns are periodically necessary in every country. This is particularly important to prevent population of bacteria resistant to antibiotics and select antibiotic of choice for particular condition. Study conducted in France to compare vitro activity of Cefoxitin with Metronidazole and Clindamycin against 322 strains of anaerobic bacteria collected from several hospitals during 1982 and tested by an agar dilution method showed that Metronidazole and Cefoxitin inhibited at least 89% of strains tested, whereas Clindamycin was less active (Dubreuil et al, 1984).

Chapter III

Objective of the study

3.1 General objective

To assess disinfectant and antibiotic resistance pattern of the pathogenic bacteria released to the environment by hospital effluents

3.2 Specific objectives

To determine total heterotrophic bacteria, total coliform, fecal coliform, *E. coli*, *enterococci*, *Pseudomonas spp*. and *Staphylococcus spp*. from hospital wastewater

To isolate common pathogenic bacteria in hospital wastewater

To assess bacterial isolates resistance pattern to common disinfectants/antiseptics and antibiotics

To compare level of disinfectant and antibiotic resistance between hospital effluents

Chapter IV

Materials and Methods

4.1 Study design

A cross sectional study design was employed and hospital wastewater samples were collected at different times during study period.

4.2 Study area and period

The study was carried out in Yirg Alem and Hawassa University Referral Hospital (HURH), which were 311 and 275 kms respectively from Addis Ababa, capital of Ethiopia. Both hospitals were located in southern Nations, Nationalities and Peoples' Region (SNNPR) and serving for more than 1,500,000 people each. They provide a range of health services to the region and neighboring regions.

Yirg Alem hospital was established in 1967 by Norwegian fund is found in Dale Woreda, Sidama Zone. People served in this hospital may come from; Bale Zone, Arisi Zone, Dolemena, Borena Zone, Kibremengist, Yavelo, Omega, Hagermariam, Gideo Zone, Burgi, Amaro & Sidama Zone. The hospital serves as a public health center that provides hospital care in a broad category of illnesses and injuries. It operates with 200 licensed beds and uses all available beds for patient management. The hospital laboratory conducts tests in the following areas: hematology, chemistry, urinalysis, microbiology & blood bank for both in-patient and outpatient services. As disinfection process it uses the following chemicals: savlon, sodium hypochlorite, alcohol (ethanol), hydrogen peroxide, iodine, formalin & phenol. Antibiotics such as Ceftiraxone, Ampicillin, Chloramphenicol, Cloxacillin, Penicillin, Amoxicillin, Methicillin, Tetracycline, Doxycycline and Gentamycin have commonly been prescribed for inpatient care. The hospital releases approximately 125 cubic meters of partially treated (pretreatment septic tank) effluents per day to open field which finally join to Gidawo river after flowing approximately 50 meters inclined land. Gidawo river is one of the largest river in Dale Woreda, Sidama Zone, SNNPR. The hospital has previously physical wastewater treatment plant (sand filter) but now it becomes non-functional.

Hawassa University Referral Hospital (HURH) is a teaching and public health center established in 2005. It is the only referral hospital in the region and serves people of the region and neighboring regions. Like Yirg Alem hospital it provides wide range of healthcare services and in addition it has been backbone to train medical students and other health professionals.

The hospital is located near to Lake Hawassa and operates with 350 licensed beds (maximum number of beds that the facility can operate) and approximately 305set-up beds (number of actual beds that are in operation and available for patient use including 18 beds in emergency ward). Hospital laboratory conducts tests similar to Yirg Alem hospital. As hospital infection control program, it uses the following chemicals: alcohol, sodium hypochlorite, iodine, formalin and savlon. Antibiotics such as Ceftriaxone, Ampicillin, Chloramphenicol, Cloxacillin, Penicillin, Amoxicillin, Methicillin, Tetracycline, Doxycycline and Gentamycin have commonly been prescribed for inpatient care. The wastewater treatment system employed in the hospital has been oxidation pond system which comprises of two facultative ponds and two maturation ponds for the treatment of the wastewater, and another additional fish pond for fish farming. The facultative ponds receive a combination of settled wastewater from a septic tank pretreatment and raw wastewater from student's dormitory and staff residence buildings. The retention time in the facultative ponds, in the first maturation pond, second maturation pond and the fishpond were 13.97, 3.08, 3.04, 9.09 days respectively. As a result, the total time the wastewater spent in the treatment system is 29.18 days. This value indicates that the detention time of the facultative pond was found to be less than the typical detention time in the facultative pond which is 25-180 days, which resulted in inefficient treatment of the wastewater. The last step in treatment process is release of approximately 143.3 cubic meters of treated effluents per day which joins Lake Hawassa after flowing approximately 20 meters sewage system. In both hospitals the wastewater were allowed in continuous flow. The study was conducted from December 2010-Febrwary 2011.

4.3 Sampling

The hospital wastewater sample was collected from Yirg Alem hospital just at the tip of drainage tube before released to open field. Effluent from Hawassa University referral hospital was collected just at the tip of drainage tube to sewage line which finally joins Lake Hawassa. Similarly influent from HURH collected from central drainage tube before entering to treatment plant (oxidation pond). A total of 96 wastewater samples were collected every two hours difference starting from the first sample at 8:30 AM for a period of 6 hours and finally 24 composite samples were used on weekly basis for bacteriological analysis and susceptibility testing.

4.3.1 Sample collection

A total of 24 composite hospital wastewater samples were collected from Hawassa University referral hospital (8 influent & 8 effluent samples) and 8 effluent samples from Yirg Alem hospital on weekly basis during the period from December 2010 to February 2011. Each partial sample were collected at 8:30AM, 4:30AM, 6:30AM & 2:30PM in small sterile bottle according to method used by (Nuñez and Moretton, 2007) and transferred into 250mL sized sterile bottle containing 0.2 mL of 3% w/v sodium thiosulphate and then transported within 2 hours in ice jackets with ice box to microbiology laboratory for analysis.

4.3.2. Sample transport and storage

Sample containing bottle was transported in ice box to analytical laboratory within 2 hours of collection. Then it was stored in refrigerator at 4^{o}C until analysis. All the samples were analyzed on day of collection.

4.4 Bacteriological characterization of wastewater

Bacteriological analysis of hospital wastewater sample and susceptibility testing were performed in microbiology and Parasitology laboratory unit, Hawassa University.

Total heterotrophic plate count- Serial 10-fold dilutions of samples were prepared in physiological saline, and 0.5mL aliquot was streak plated on tryptone glucose yeast agar (TGYA). Plates were incubated for 48 h at 37ºC before bacteriological counts were preformed. Number of colonies on duplicate plates having 30-300 colonies was counted by using digital colony counter. Finally bacterial count reported CFU/ mL as follows:

$$\text{CFU/mL}= \frac{colonies\ \ counted}{Actual\ \ volume\ \ of\ \ sample\ \ in\ plate\ ,mL}$$

***Staphylococcus* count:** Appropriate dilutions were prepared and 0.5 mL of aliquot was streak plated on mannitol salt agar (MSA) and incubated at 37^{o}C for 24-48 h. Colonies showing a typical yellow zone of fermentation were isolated for Gram staining. Those colonies identified as gram-positive cocci were counted using digital colony counter as *Staphylococci* according to method used by (Dudely et al, 1980). Similar reporting as above was applied to determine the CFU/mL.

***Total coliform count**:* Serial 10-fold dilutions of sample were prepared in physiological saline and 1mL of aliquot was transferred aseptically in to series of test tubes containing Durham tube and lauryl tryptose broth (LTB). Tubes were gently shaken and incubated for 48h at 37ºC, then production of gas and lactose fermentation was observed as positive reaction.

Fecal coliform count: Serial 10-fold dilutions of sample were prepared in physiological saline and 1mL of aliquot was transferred aseptically in to series of test tubes containing Durham tube and lauryl tryptose broth. Tubes were gently shaken and incubated for 48h at 44.5ºC, then production of gas and lactose fermentation was observed as positive reaction.

Escherichia coli: Serial 10-fold dilutions of sample were prepared in physiological saline and 1mL of aliquot was transferred aseptically in to series of test tubes containing *Escherichia coli* 4-methylumbelliferyl-β-glucuronide (EC-MUG) medium. Tubes were gently shaken and incubated for 48h at 44.5ºC, then all tubes examined for growth of bright blue fluorescence using long wavelength UV lamp which was considered a positive response for *E. coli*.

***Enterococci* count:** Serial 10-fold dilution of sample was prepared in physiological saline and 1mL of aliquot was transferred aseptically in to series of test tubes containing brain heart infusion broth (BHIB). Tubes were gently shaken and incubated for 48h at 44.5ºC, then all tubes examined for turbidity and considered as positive.

All methods were used according to standard methods for examination of water and wastewater, APHA, 1999.

For all tube methods, bacterial load were estimated using most probable number (MPN) and reported as MPN/100mL as follows:

$$\text{MPN/100mL} = \frac{Number\ of\ positive\ tubes\ x100}{\sqrt{mL}\ sample\ in\ negative\ tubes\ x\ \sqrt{mL}\ sample\ in\ all\ tubes}$$

Identification of common pathogenic bacteria: Most important pathogenic bacteria found in hospital wastewater were identified 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, APHA 1999.

4.5 Antimicrobial susceptibility testing

4.5.1 Disinfectant susceptibility testing

Activities of the following disinfectants in use-dilution were determined:

Tincture-iodine: 5mL of effluent sample were treated with 0.1% of 5mL tincture-iodine for 5 minutes in sterile test tube. Then 0.5mL of aliquot was streak plated on nutrient agar and incubated for 48h at 37ºC. Finally growth of colonies was observed and if any present, bacteria were identified and MIC was determined according to Hani and Adnan, 2009.

***Sodium hypochlorite*:** 5mL of effluent sample were treated with 0.5% of 5mL sodium hypochlorite for 5 minutes in sterile test tube. Then 0.5mL of aliquot was streak plated on nutrient agar and incubated for 48h at 37ºC. Finally growth of colonies was observed and if any present, bacteria were identified and MIC was determined according to Hani and Adnan, 2009.

70% ethanol (ethyl alcohol): 5mL of effluent sample were treated with 70% of 5mL ethanol for 5 minutes in sterile test tube. Then 0.5mL of aliquot was streak plated on nutrient agar and incubated for 48h at 37ºC. Finally growth of colonies was observed and if any present, bacteria were identified and MIC was determined according to Hani and Adnan, 2009.

Minimum inhibitory concentration (MIC) for 70% ethanol resistant organisms was determined as follows:

MIC values of ethanol were determined on tubes containing 1mL of 60%, 65%, 70%, 75% and 80% of ethanol. One mL of bacterial suspension with concentration approximately 1 X 10^6/mL were transferred in to each tube and treated with different concentration of ethanol for 5 minutes. Then nutrient agar plates were inoculated with 0.5mL of treated suspension. All plates were incubated for 48 h at 37ºC and the number of colonies was counted. The MIC was the lowest concentration that prevented bacterial growth (Murray et al, 2003). Bactericide activities were expressed as reduction factors, that is, logarithmic reductions in viable organisms: Reduction factor = $\log_{10}$ CFU/mL $_{(\text{negative control})}$ - $\log_{10}$ CFU/mL $_{(\text{treated})}$

4.5.2 Antibiotic susceptibility testing

The following antibiotics were commonly prescribed in these two Hospitals: Ceftriaxone, Ciprofloxacin, Ampicillin, Gentamycin, Doxycycline, Amoxicillin, Tetracycline, Vancomycin & Penicillin. The susceptibility of pathogenic and potentially pathogenic isolates to these antibiotics was determined by Kirby-Bauer disk diffusion method. All the antibiotic disks used were from Oxoid Company. A sterile swab were dipped in a bacterial suspension (McFarland standard 0.5, which approximate bacterial population of $1X10^6$ CFU/mL) and were streaked onto Müeller Hinton Agar. Antibiotic disks were applied using a sterile forceps. Agar plates were incubated at 37°C for 18h and the zone of inhibition was measured in millimeters using ruler. Interpretation was made using susceptibility breakpoints annually published by the National Committee for Clinical and Laboratory Standards Institute (CLSI).

4.6 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, antibiotic disks & disinfectant solution were insured following the manufacturer's direction. In addition, reference strains (quality control strains) were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI) and employed 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.

4.7 Data analysis

Data were entered, cleaned and analyzed using SPSS v16.0 Statistical Software. Descriptive statistics were 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. Pair wise comparisons while applying ANOVA was done using Bonferroni's test. A critical value of 0.05 was used for the inferential statistics.

4.8 Ethical considerations

This thesis work has got ethical clearance from DMIP ethical committee of the Medical School of AAU. Permission were sought from concerned authorities such as SNNPR Regional Bureau of Health, Hospital administrative bodies and Environmental Protection Agency using letter of justification owned from Department of Medical Microbiology, Immunology and Parasitology, AAU.

CHAPTER V

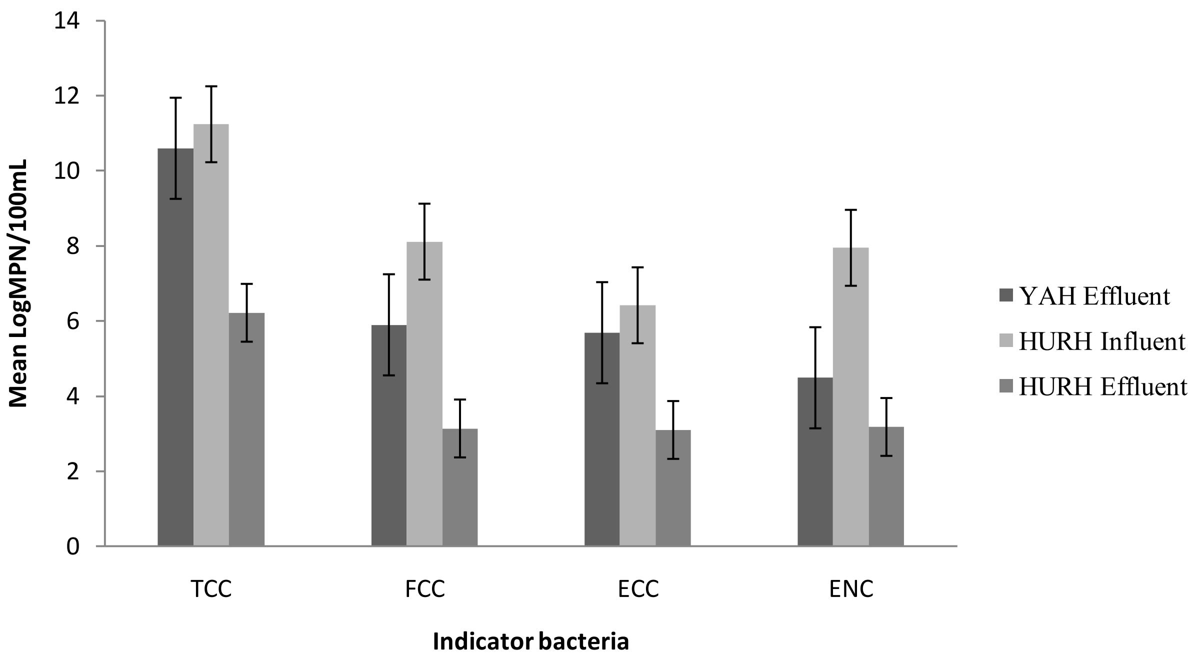
Results

A total of 24 composite samples (eight influents from HURH, eight effluents from HURH and eight effluents from YAH) were collected on weekly basis for a period of two months for microbiological analysis. All samples were preserved at $4^{o}C$ in refrigerator until analysis and processed on day of collection. Average results of each test parameter were displayed accordingly.

Wastewater quality indicator organism enumeration was made using tube and plate method after serial dilution of the sample in physiological saline. Appropriate volume of wastewater was inoculated in test media. After overnight incubation bacterial concentration were determined. Table 1 shows average number of indicator bacteria in sampling sites with HURH influent was highest compared to HURH and YAH effluent.

Table 1. Geometric mean of indicator organism in hospital wastewater, 2010/2011

Indicator organism	*Sample site*		
	HURH influent	*HURH effluent*	*YAH effluent*
Heterotrophic plate count CFU/mL	$2.1 X 10^{6}$	$5.0 X 10^{5}$	$5.2 X 10^{6}$
Staphylococcal count CFU/mL	$2.5 X 10^{3}$	$2.0 X 10^{3}$	$2.3 X 10^{3}$
Total coliform count MPN/100mL	$1.7 X 10^{11}$	$1.6 X 10^{6}$	$4.2 X 10^{10}$
Fecal coliform count MPN/100mL	$1.4 X 10^{8}$	$1.4 X 10^{3}$	$8.0 X 10^{5}$
E. coli count MPN/100mL	$2.6 X 10^{6}$	$1.2 X 10^{3}$	$4.8 X 10^{5}$
Enterococci count MPN/100mL	$9.0 X 10^{7}$	$1.5 X 10^{3}$	$8.6 X 10^{4}$



Log_TCC-logarithm of total coliform count, Log_FCC-logarithm of fecal coliform count, Log_ECC-logarithm of E. coli count, Log_ENC-logarithm of enterococcal count

Figure 1. Mean indicator bacterial concentration (log_{10}-transformed) in hospital wastewater, log MPN/100 mL, 2010/2011

A total of 16 samples, 8 influent and 8 effluent samples were tested for indicator organisms before and after treatment of wastewater in Hawassa University referral hospital. The result showed in the table indicates average percent reduction of organisms by wastewater treatment plant (oxidation pond).

Table 2. Reduction of indicator bacteria by wastewater treatment plant (oxidation pond), HURH, 2010/2011

Indicator/bacteria	*Influent*	*Effluent*	*Percent (%) of reduction*
Heterotrophic plate count CFU/mL	2.1×10^{6}	5.0×10^{5}	76.19
Total coliform count MPN/100mL	1.7×10^{11}	1.6×10^{6}	99.99
Fecal coliform count MPN/100mL	1.4×10^{8}	1.4×10^{3}	99.99
E. coli count MPN/100mL	2.6×10^{6}	1.2×10^{3}	99.95
Enterococci count MPN/100mL	9.0×10^{7}	1.5×10^{3}	99.99

The study also revealed presence of variety of organisms including pathogenic and non pathogenic (environmental) bacteria in wastewater. They were found in high concentration and frequently detected. The most commonly identified groups were: *Staphylococcus spp, Klebsiella spp, E. coli, Bacillus spp, Proteus spp, Enterococci spp, Salmonella spp, Shigella spp, Citrobacter spp* and unidentified gram negative rods.

Medically important pathogenic bacteria like *S. aureus*, *Salmonella* and *Shigella* from wastewater of both hospitals were identified after culturing on selective bacteriologic media. *S. aureus* was found in high concentration of all sampling sites. The rate of detection and identification of *Salmonella* was higher compared to *Shigella* in all sites. The relative detection of these organisms was presented in the figure below.

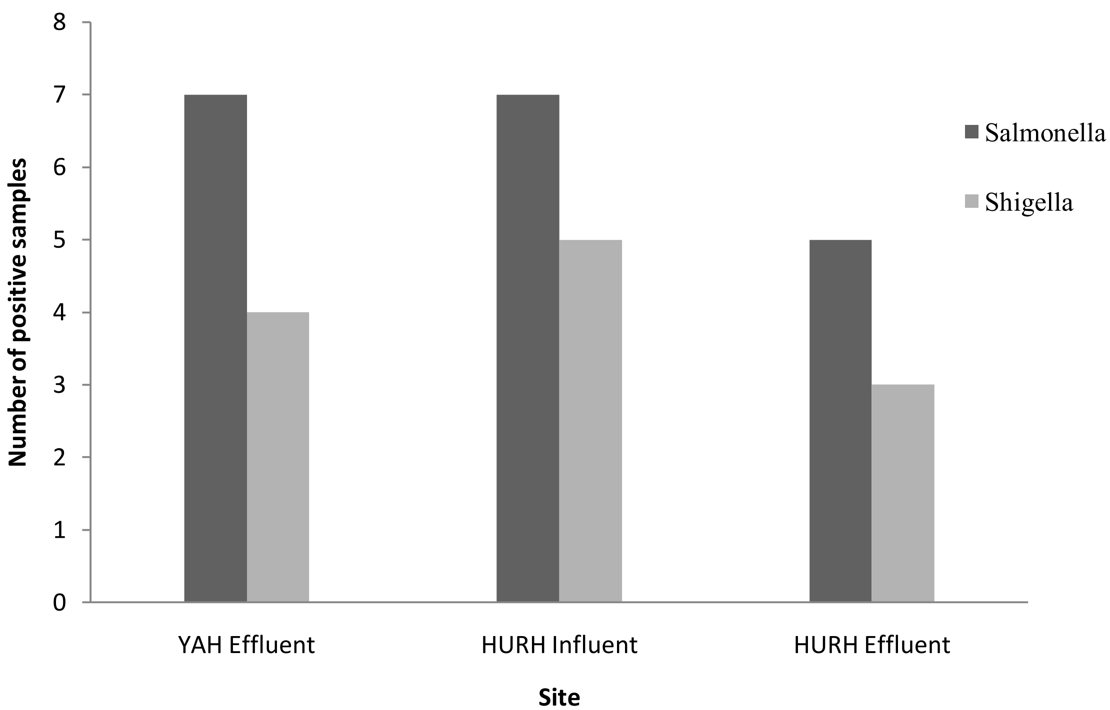


Figure 2. Frequency of detection and identification of *Salmonella* and *Shigella* in weekly base sampled hospital wastewater, 2010/2011.

Wastewater treatment plant is important not only for reduction of indicator organisms, but also pathogenic bacteria. Although the rate of reduction of pathogenic bacteria much lower as compared to indicator organisms, the highest reduction was observed in *E. coli* followed by *Shigella*.

Table 3. Percent reduction of pathogenic and potential pathogenic bacteria by wastewater treatment plant (oxidation pond), HURH, 2010/2011

Organism detection/concentration	*Influent*	*Effluent*	*% reduction*
Salmonella	7	5	25.6
Shigella	5	3	40
Staphylococcal count CFU/mL	2.5×10^3	2.0×10^3	20.00
E. coli count MPN/100ml	2.6×10^6	1.2×10^3	99.95

Stock solution of disinfectant (ethyl alcohol and sodium hypochlorite) obtained was prepared in use-dilution according to manufacturer's direction. Then hospital effluent was treated with equal volume of disinfectant solution and streak plated on nutrient agar. Finally appearance of if any colony on the plate was observed and further identified. Then relative effectiveness of disinfectant solutions was determined by absence or presence of resistant bacteria on plate. Tincture iodine treated effluent showed no growth in all samples. *Bacillus species* were frequently isolated from plates treated with sodium hypochlorite. *Bacillus species*, Gram negative rods and *S. aureus* were frequently isolated as resistant for 70% ethanol, which was the least effective.

Table 4. Evaluation of disinfectant activities against hospital wastewater organisms, 2010/2011

Disinfectant	*effluent site*		
	YAH	*HURH*	*Resistant bacteria isolated*
Tincture iodine	*Effective**	*Effective*	*No resistance*
Sodium hypochlorite	*Effective*	*Effective*	*Bacillus spp*
Ethanol 70%	*Less effective*†	*Less effective*	*S. aureus, Bacillus spp, Gram negative rods*

* -no growth observed on plate, † -growth observed on plates

Minimum inhibitor concentration (MIC) of ethanol was determined using different concentration. Two-three colonies of 70% ethanol resistant *S. aureus* and gram negative rods were inoculated in nutrient broth and incubated at 37 ^{O}C for 24 h. Then bacterial suspension of approximately 1 X 10^6 CFU/mL was treated with different concentration of ethanol. The results showed that ethanol inhibitor effect/killing power was significantly reduced at 60% and 80%. Log reduction was calculated by subtracting number of CFU from negative control. The optimum concentration which resulted in highest log reduction for isolates of HURH effluent was 70%, with 3.8 and 3.2 log reduction for *S. aureus* and gram negative rod bacteria respectively. Similarly the optimum concentration which resulted in highest log reduction for isolates of YAH effluent was 70%, with 4.0 and 3.5 log reduction for *S. aureus* and gram negative rod bacteria respectively. The bell shaped pictures (picture 3 and 4) below demonstrated relationship of log reduction with ethanol concentration.

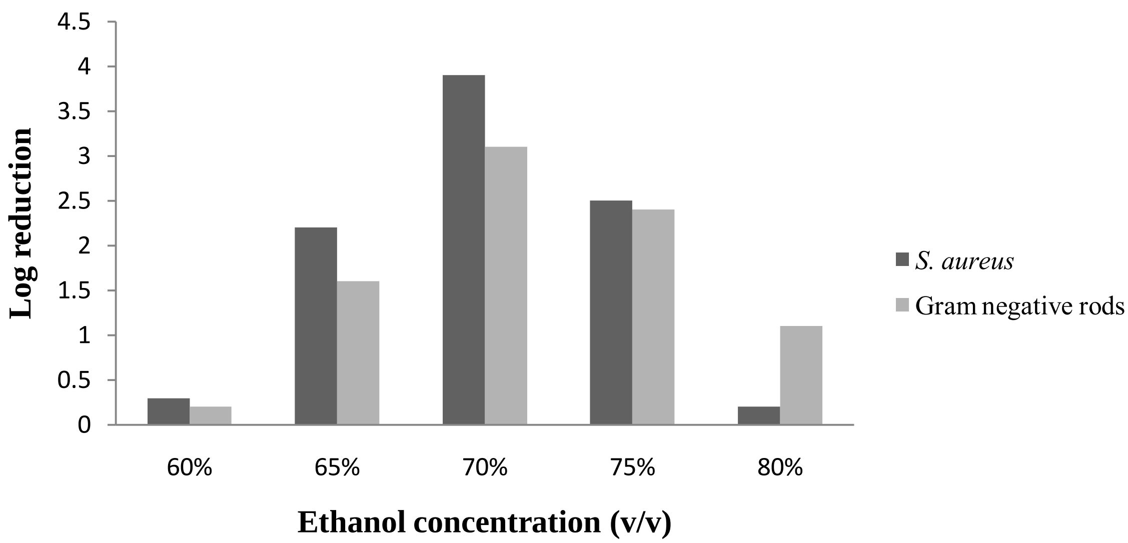


Figure 3. Determination of MIC of ethanol against *S. aureus* and gram negative rods, HURH effluent, 2010/2011.

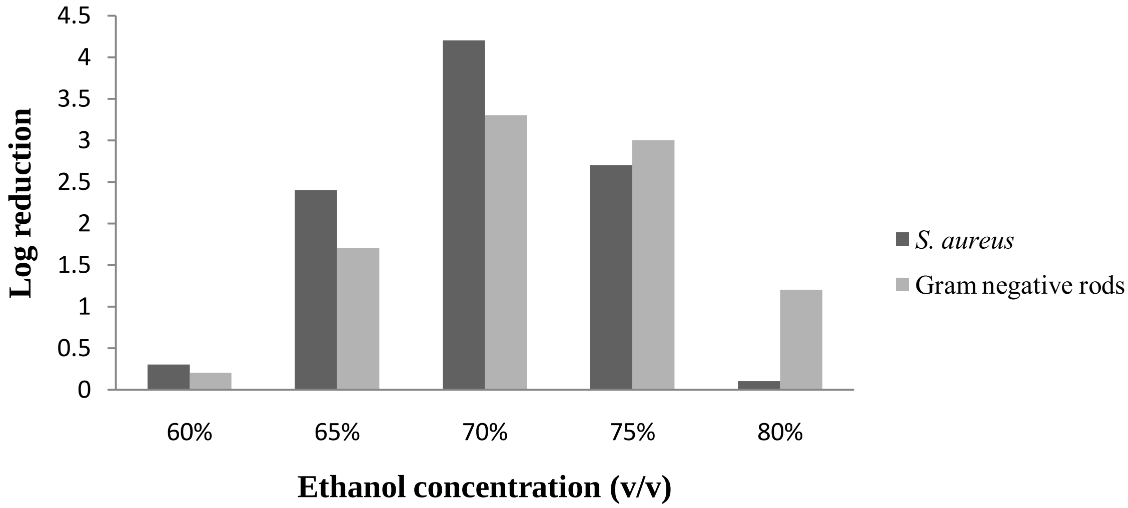


Figure 4. Determination of MIC of ethanol against *S. aureus* and gram negative rods, YAH effluent, 2010/2011

Susceptibility of medically important bacteria (*S. aureus*, *Salmonella*, *Shigella* and *E. coli*) to commonly prescribed antibiotics was determined using Kirby-Bauer disk diffusion methods. The zone of inhibition were measured in millimeters and interpreted as susceptible or resistant according to susceptibility breakpoints of national committee for Clinical and Laboratory Standards Institute (CLSI). Patterns of susceptibility to different antibiotics have shown below in the tables 5, 6 and 7.

Table 5. Antibiotic susceptibility pattern of *Salmonella*, *Shigella* and *E. coli* isolated from effluents of YAH, 2010/2011

Antibiotic /concentration♠	*Organism tested*		
	Salmonella spp	*Shigella spp*	*E. coli*
Amoxicillin (30)	S	S	S
Ciprofloxacin (1)	S	S	S
Ceftriaxone	R	S	R
Tetracycline (10)	R	R	R
Gentamycin (10)	S	S	R
Doxycycline (30)	R	R	R

S- susceptible, **R**-resistant, ♠ -µg

Table 6. Antibiotic susceptibility pattern of *Salmonella*, *Shigella* and *E. coli* isolated from effluents of HURH, 2010/2011

Antibiotic/concentration♠	*Organism tested*		
	Salmonella spp	*Shigella spp*	*E. coli*
Amoxicillin (30)	S	S	S
Ciprofloxacin (1)	S	S	S
Ceftriaxone	R	S	R
Tetracycline (10)	R	R	R
Gentamycin(10)	R	S	R
Doxycycline (30)	R	S	R

S-susceptible, **R**-resistant, ♠ -µg

Table 7. Antibiotic susceptibility pattern of *S. aureus* isolated from YAH and HURH effluents, 2010/2011

Antibiotic/concentration♠	*Hospital effluent site*	
	YAH	*HURH*
Gentamycin (10)	S	R
Erythromycin	S	S
Penicillin (10)	R	R
Vancomycin (30)	S	S
Ampicillin (25)	R	R
Amoxicillin (30)	R	R
Ciprofloxacin (1)	S	S

S-susceptible, **R**-resistant, ♠ -µg

CHAPTER VI

Discussion

Bacteriological analysis of water and wastewater are used to assess its quality for human consumption, recreational purpose or agricultural activities to safe guard public health. To our knowledge, this is the first study in Ethiopia, which has attempted to assess microbiological quality of hospital effluent, detection of some pathogenic bacteria and determine disinfectant and antibiotic susceptibility of some bacterial isolates.

All indicator and pathogenic organisms tested for YAH effluent were higher than HURH effluent. Mean heterotrophic plate count was found 5.2 X 10^6, 2.1 X 10^6, 5.0 X 10^5 CFU/mL for YAH effluent, HURH influent and HURH effluent respectively. There was no significant statistical difference among the different sites ($p>0.05$). Comparisons of effluent quality of the two sites and with other similar studies have been done. Our finding correlate with the results of University of Benin teaching hospital wastewater, Nigeria which were 1.9 X 10^7 CFU/mL (Ekhaise and Omavwoya , 2008). Comparable results were reported from Nepal hospitals which discharge treated effluent with 2.9 X 10^7 and 4.0 X 10^7 CFU/mL and in hospital which discharge raw sewage 3.2 X 10^7 CFU/mL (Sharma et al, 2010). Lower results 1.8 X 10^5 CFU/mL were reported in Buenos Aires City hospital wastewater, Brazil (Nuñez & Moretton, 2007). Heterotrophic plate counts from both hospitals were beyond WHO standard limit and warn possible presence of pathogenic organisms in hospital effluents.

Mean Staphylococcal count were found 2.3 X 10^3, 2.5 X 10^3, 2.0 X 10^3 CFU/mL at YAH effluent, HURH influent and HURH effluent respectively. There was no significant statistical difference among the three sites ($p>0.05$). Much lower results reported from University of Benin teaching hospital wastewater, Nigeria which was 85 CFU/mL (Ekhaise and Omavwoya, 2008). Results of this study showed presence of high number of *staphylococcus* in hospital effluents. Presence of staphylococcus species in hospital effluents which is discharged in to lake and river pose risk to the public health associated with staphylococcal infection and warns public health authorities to take proper intervention.

The geometric mean of total coliform count were 4.2 X 10^{10}, 1.7 X 10^{11} & 1.6 X 10^{6} MPN/100mL for YAH effluent, HURH influent and HURH effluent respectively. The result showed statistically significant difference between HURH influent and effluent ($p<0.01$). There was also statistically significant difference between YAH effluent and HURH effluent ($p<0.01$). Relatively low number of total coliform in HURH effluent may be due to reduction of coliform organisms in the treatment process of wastewater. Finding of 2.2 X 10^{7} MPN/100mL comparable to HURH effluent were reported from Razi hospital having activated sludge processing treatment plant and 3.8 X 10^{8} MPN/100mL from Bahrami hospital which discharge their wastewater into municipal wastewater collection system at second phase of Tehran sewerage project, Iran (Mesdaghinia et al, 2009). Treatment process will reduce toxic chemical compound, infectious organisms and indicator bacterial load from the sewage system. Study conducted in North Dakota showed mean coliform count; in raw influent 3.2 X 10^{7}, in primary lagoon 9.3 X 10^{5} and in secondary lagoon 8.8 X 10^{4} CFU/100mL from domestic and oxidation lagoon (Micheal and John, 1985). Moreover (Rezaee et al, 2005) reported significant reduction of chemical oxygen demand (COD) 95.1%, coliform (90%) and *E. coli* (98%) using integrated anaerobic aerobic fixed film bioreactor wastewater treatment plant. Similarly (Silva et al, 2006) reported 80–90% reduction of heterotrophs and total coliform as well as 84-96% reduction of fecal coliform and *enterococci* following urban wastewater treatment. However (Silva et al, 2007) reported wastewater treatment led to a slight decrease in the relative proportions of *Escherichia spp*. isolates, with a concomitant increase in *Acinetobacter, Shigella* and *Klebsiella*.

Presence of high level total coliform in YAH effluent was indicative for presence of pathogenic bacteria, which is released in to open field and finally joins Gidawo river. Moreover bacteria from hospital effluent are usually multiply drug resistant and infections caused by these organisms are difficult to antibiotic treatment.

Comparing geometric mean fecal coliform counts recorded for the three sites revealed that the highest Hawassa influent 1.4 X 10^{8} followed by Yirg Alem effluent 8.0 X 10^{5} and the lowest Hawassa effluent 1.4 X 10^{3} MPN/100mL. There were statistical significant difference among the three sites ($p<0.05$). This is in line with the findings of similar study by (Nuñez & Moretton, 2007).WHO has indicated maximum tolerable limit for fecal indicator bacteria ($\leq 10^{3}$/100mL for unrestricted irrigation and $\leq 10^{5}$/100mL for restricted irrigation) present in treated wastewater that has been used in agriculture (WHO, 2000). Still the fecal coliforms are higher as total coliform for YAH effluent and indicative for presence of pathogenic bacteria in wastewater which is released in to receiving environment and pose public health threat.

The geometric mean of *E. coli* count were found 4.8 X 10^5, 2.6 X 10^6 & 1.2 X 10^3 MPN/100mL for YAH effluent, HURH influent and HURH effluent respectively. Similarly mean *Enterococcal* count were 8.6 X 10^4, 9.0 X 10^7 & 1.5 X 10^3 MPN/100mL for YAH effluent, HURH influent and HURH effluent respectively. This is also in line with the findings of similar study by (Nuñez & Moretton, 2007).

As summary, indicator organisms released from YAH effluent were much higher as compared to HURH. This may be due to absence of wastewater treatment plant in the case of YAH and other factors. In general factors like hospital water consumption, type of disinfectant used, number of beds, range of laboratory tests performed, availability of wastewater treatment facility etc will determine number of pathogenic and indicator organism released which were not considered in this study.

In this study, some of pathogenic and potentially pathogenic bacteria were detected otherwise diverse group of bacteria were reported in literature reviewed. Organisms were *Salmonella species, Shigella species, Staphylococcus aureus, & E. coli. Staphylococcus aureus* and *E. coli* were detected in high concentration from both hospital effluents as indicated in table 1.There were variation in detection of *salmonella* and *shigella*. Seven samples out of eight (7/8) and five samples out of eight (5/8) were positive for *salmonella* from YAH and HURH effluent, respectively. Similarly four samples out of eight (4/8) and three samples out of eight (3/8) were positive for *shigella* from YAH and HURH effluent, respectively. However, (Dudley et al, 1980) reported variety of pathogenic and potentially pathogenic bacteria in sewage sludge with *shigella* none detected due to low sensitivity of enrichment procedure and high temperature decreasing survival. High frequency of detection of pathogenic bacteria in our study may be due to admission of cases with these bacterial infections, which are common in developing country like Ethiopia. Study conducted by (Somwang et al, 2005) reported microbial and heavy metal contamination of treated HWW with diverse group of organisms: pathogenic, potentially pathogenic bacteria and parasites. Similarly (Julia Baudart et al, 2000) reported the highest numbers of *salmonella* isolates were obtained from samples collected in the river and in wastewater. Although the number of samples in this study small, lesser detection of *Salmonella* and *shigella* in HURH effluent may be due to presence of wastewater treatment plant. Again (Momba et al, 2006) reported gradual removal of presumptive bacterial pathogens in different zones of treatment plants. In his findings, there were variation with regards to both the patterns and efficiency of each plant for the removal of target pathogen, about 71% of the total influent samples contained presumptive *Salmonella*, while only 50-33.5% of the effluent and receiving water body samples were observed to contain presumptive *Salmonella*. Similar observations were made for presumptive *Shigella* and *Vibrio* pathogens with decreasing incidence of pathogens from influents to the receiving water bodies (Somwang et al, 2005).

Staphylococcus aureus were detected in high number in all effluent samples from both hospitals and continuously released to receiving environment. This is in line with other studies which reported that the organism is resistant to antiseptics, disinfectant and antibiotic and survive in the sewage system for long period (Nuñez & Moretton, 2007; Ekhaise and Omavwoya, 2008; Martin et al, 1966; and 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 findings of our study showed that in the presence of wastewater treatment facility in HURH, proportional amount of pathogenic bacteria were detected as compared to YAH. This may be due to inefficient removal of pathogenic bacteria by oxidation pond or admission of large number of cases with these bacterial infections.

Although most *Escherichia coli* strains are non pathogenic to human and exist as normal flora of human and animal gut, some strains are associated with gastrointestinal disease. We suspect presence of diarrhogenic strains from both hospital effluents which were reported in similar studies in different countries.

Although bacterial resistance to antibiotics has been extensively studied in both clinical and environmental samples, only a few reports were available on disinfectant activity against microorganisms. Susceptibility pattern of organisms in effluent sample tested to disinfectant solutions in-use dilution: 0.1% tincture iodine, sodium hypochlorite with 0.5% free chlorine and 70% ethanol were evaluated. These chemicals are routinely used for antiseptics and disinfection process in the hospitals. Iodine has long been considered an effective antimicrobial agent, especially when used in the form of providone-iodine (Nuñez & Moretton 2007). This study revealed that tincture iodine treated effluent showed no growth on nutrient agar after incubation. Our results supported by (Favero and Drake, 1966) which reported iodine as efficient microbicide compared to chlorine in swimming pool. However, (Nuñez and Moretton, 2007) reported iodine resistant organisms such as *S. epidermidis*, *Bacillus spp* and *Pseudomonas alcaligenes* and resistance increased in nutrient restricted environment like oligotropic aquatic system found in HWW.

In sodium hypochlorite (with 0.5% free chlorine) treated effluent, *Bacillus spp* was resistant and grown in plate. Although this organism is less important in HWW, it was frequently detected in treated wastewater. Similar observations were reported in different studies (Russell, 2002; and Favero and Drake, 1966). However comparative study conducted in Brazil to compare disinfectant activity against standard strain, antibiotic susceptible and resistant hospital strain revealed sodium hypochlorite effective against all strains tested (Aparecida et al, 2000).

Alcohols exhibit rapid broad-spectrum antimicrobial activity against vegetative bacteria (including mycobacteria), viruses, and fungi but are not sporicidal (Russell, 2002).The least activity were observed in Hospital effluent treated with 70% ethanol (ethyl alcohol). Resistant organisms commonly isolated were *S. aureus, Bacillus spp* and gram negative rods. Study conducted by (Suzanne et al, 2002) explained resistance of *S. aureus* due to biofilm formation; biofilm cells were less susceptible to disinfectants (sodium hypochlorite and benzalkonium chloride) than suspension test cells. Also spores of bacillus may resist ethanol treatment and sporulation can occur in nutrient rich medium. Gram negative bacteria are generally resistant to antiseptics, disinfectant and antibiotics because of their special cell wall, outer membrane.

The minimum inhibitor concentration for resistant bacteria was determined using different concentration (60%, 65%, 70%, 75% and 80%) of ethanol and result showed 70% remain as minimum inhibitor concentration for *S. aureus* and gram negative rods with log reduction of 4 and 3.5 for isolates from YAH, respectively. Similarly 3.8 and 3.2 log reduction was observed for *S. aureus* and gram negative rods from HURH, respectively. There was no significant statistical difference of disinfectant resistance pattern and MIC value for isolates of both hospitals ($p>0.05$). Furthermore, 70% of ethanol was able to kill/inhibit more than 50% of the inoculum and can be used for routine disinfection/antiseptic purpose in the hospitals.

Resistance may be acquired in the community, hospital environment or in sewage system. Especially the sewage system allows interaction of diverse group of organisms resulting transfer of drug resistant genetic elements among different bacterial population. The overall effect of development of resistant bacteria in the community could lead to failure to respond for available antibiotics and decrease our antibiotic pool for treatment of bacterial infection.

Antibiotic susceptibility test results for: Penicillin, Ampicillin, Tetracycline, Vancomycin, Gentamycin, Ciprofloxacin, Erythromycin, Doxycycline and Ceftriaxone against important organisms displayed in the table 5, 6 and 7. Direct comparison of our findings with other scholars was difficult because of differences in type of antibiotic used; type of wastewater sample collected and type of organism isolated in most literature were different from our studies. Susceptibility pattern (table 5 & 6) against *E. coli* revealed that the organism was resistant to Ceftriaxone, Tetracycline, Gentamycin and Doxycycline. Similar study conducted in Bangladesh showed that all *E. coli* isolates from untreated HWW were multi-drug resistant (≥4) and were resistant to Tetracycline (100%), Ciprofloxacin (100%), Penicillin (100%), Erythromycin (100%), Gentamycin (50%) and Chloramphenicol (90%) but all of them were sensitive to Imipenem (Islam et al, 2008). Another study conducted in India showed that *E. coli* in HWW were resistant to Amikacin and sensitive to Amoxicillin (Vishal et al, 2010). Again (Watkinson et al, 2007) reported the highest incidence of *E. coli* resistance for tetracycline (51%), followed by those for Cephalothin (41%) and Sulfafurazole (32%). Another study from Ireland showed that high proportion of *E. coli* discharged from secondary wastewater treatment facilities is resistant to Ampicillin and that isolates resistant to newer agents such as extended-spectrum Cephalosporins (including the ESBL phenotype) and fluoroquinolones are also present (Sandra et al, 2010).

Data regarding *shigella* from hospital effluent is very scarce and comparisons of these findings become difficult. This is because the organism itself rarely recovered from sewage sample and its survival in fresh water and sewage is less than 30 days (WHO, 2006). Here in this study, *Shigella* species were detected in effluent samples of both hospitals. The organism is highly associated with diarrheal disease, especially in children of developing countries. Study conducted in Greece showed that based on epidemiologic and environmental findings, transmission of shigellosis was waterborne due to contamination of spring by untreated sewage effluent (Samonis et al, 1994). Findings of this study showed that *shigella* isolates of YAH effluent were resistant to Tetracycline and Doxycycline (table 5) and isolates from HURH effluent were resistant to Tetracycline (table 6). Study by (Silva et al 2007) reported all *shigella spp* isolated from raw sewage were susceptible for Ciprofloxacin; Gentamycin; Sulfamethoxazole/Trimethoprim; and Tetracycline, while some proportion of those isolated from treated wastewater were resistant to the same antibiotics.

Salmonella species are pathogenic to human and has multiple hosts, which is usually transmitted through food and water contamination. They cause typhoid fever, paratyphoid fever and gastroenteritis. The organism was frequently isolated from both hospital effluents. Its survival in fresh water and sewage is less than 60 days and better recovered from sewage system compared to *Shigella* (Samonis et al, 1994). Study by (Berge et al, 2006) reported *Salmonella enterica* isolates were easily recovered from municipal wastewater even in filtering volumes as small as 1 mL. Our result (table 5 and 6) showed that isolates from YAH effluent were resistant to Ceftriaxone, Tetracycline and Doxycycline. Whereas isolates from HURH effluents were resistant to the above three antibiotics and Gentamycin too. Study conducted in municipal water treatment plants in two California cities showed that a set of *Salmonella* serovars were multiple antibiotics resistant with many serovars sharing the resistant phenotypes and there was no significant difference in the levels of multiple antibiotic resistance between the two study sites (Berge et al 2006). Similarly (Alcaide and Garay, 1984) reported 12.7% of the *Salmonella* isolates from wastewater and sewage-contaminated surface waters were resistant to one or more compounds. Of these, 36.6% were resistant to one and 63.4% were resistant to two or more antibiotics and Streptomycin resistance was the most common, followed by resistance to Ampicillin. Study by (Sarina et al 2010) reported out of 36 *S. enterica* serovar typhimurium isolated from municipal wastewater, only 1 (2.8%) was sensitive to all tested antibiotics, 35 (97.2%) were resistant to one or more antibiotics, including 33 (91.7%) resistant to Ampicillin, and 18 (50%) were resistant to six antibiotics (Ampicillin, Chloramphenicol, Sulphamethoxazole, Tetracycline, Streptomycin and Kanamycin). Also (Morinigo et al, 1990) reported the highest (60%) of *salmonella* isolates of seawater affected by discharges of sewage effluent were resistant to Suphadiazine, Tetracycline or Streptomycin.

Antibiotic susceptibility pattern of *Staphylococcus* is well studied in clinical sample, however very limited report available on resistance pattern of isolates in hospital effluent. Susceptibility pattern to common antibiotics (table 7) against *S. aureus* showed resistance to Penicillin, Ampicillin and Amoxicillin for YAH effluent isolates. Isolates from HURH effluent were resistant to the above three antibiotics and Gentamycin too.

A number of studies conducted in different areas of the world indicated presence antibiotic resistant bacteria in hospital effluent and other sewage. For instance, study conducted by (Chitnis et al, 2000) reported multiple drug-resistant bacteria population in hospital effluents ranged from 0.58 to 40% for ten hospitals studied while it was less than 0.00002 to 0.025% for 11 sewage samples from the residential areas. Also (Alton et al 1969) reported approximately 1% of the lactose-fermenting bacteria found in raw and treated sewage are multiply resistant to antibiotics commonly used for the treatment of bacterial infections in man and animals. Similar study conducted in South Africa to compare drug resistance of Coliform bacteria in hospital and City sewage showed higher percent of resistance for Ampicillin, Chloramphenicol, Kanamycin, Neomycin, Oxytetracycline, Tetracycline and Sulfonamides from hospital isolates (Grabow and Prozesky, 1973). Our finding showed that pattern of resistance in isolates of both hospital effluents were almost similar, which were supported by (Chitnis et al, 2000) which reported numbers of resistant bacteria were found to be similar in both raw and treated sewage. And further indicated that no significant difference in the levels of antibiotic resistance between the two study sites.

Limitations

Although Pseudomonas is important organism in water and wastewater, this study failed to count and characterize it mainly due to lack of selective bacteriologic media. Again current study conducted for two months which was not representative for effluent discharge of varies season of the year, therefore findings of this study may not indicate actual situation in study area. *E. coli* is potentially pathogen and diarrhogenic strains were not typed mainly due to lack of biochemical test and serological kit constraints. Data relevant to wastewater quality such as amount of water employed, type of patient usually admitted etc were not considered so this limited us to interpret our findings in varies angles. Finally direct comparisons of antimicrobial susceptibility test of our findings were difficult mainly due to ***variation in antimicrobials used, type of wastewater sampled and bacterial species isolated.***

Conclusion

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.

The current study also revealed presence of pathogenic (*salmonella spp, shigella spp* and *S. aureus*) and potentially pathogenic (*E. coli*) bacteria in both hospital effluents which are highly associated with gastrointestinal disease and other infections. The concentration of *S. aureus* and *E. coli* was high in HWW sample of all sites. However; detection of *Shigella* was relatively low. This may be due to biology of the organism in wastewater and low sensitivity of enrichment procedure we were used. Presence of pathogenic bacteria in effluents of HURH may be due to inefficient removal of pathogenic bacteria by wastewater treatment process (oxidation pond). Further study will be required to proof the proper functioning of oxidation pond and if any problem investigated urgent maintenance should be done to release effluent within standard limit.

Relative disinfectant activity in use-dilution was measured and tincture iodine was the most effective, followed by sodium hypochlorite and the least active was 70% ethanol. *Bacillus spices* were commonly isolated from plates treated with sodium hypochlorite. In addition to *Bacillus spices*, *S. aureus* and gram negative rods were resistant to 70% ethanol. The MIC for ethanol resistant *S. aureus* and gram negative rods remains 70% with 4 and 3.5 log reductions for isolates from YAH. Similarly 70% ethanol remains the MIC for ethanol resistant *S. aureus* and gram negative rods with 3.8 and 3.2 log reductions for isolates from HURH. This MIC was able to kill/inhibit more than 50% of the inoculums. As the goal of disinfection is to reduce vegetative bacteria, the studied chemicals were killed most effluent bacteria when used in recommended dilution. Relative effectiveness of tincture-iodine may be due to less frequent use in studied hospitals.

This study also illustrated presence of antibiotic resistant pathogenic and potentially pathogenic bacteria in hospital effluent. *Salmonella* resistance to Ceftriaxone, Tetracycline and Doxycycline were observed for isolates from YAH effluent, while isolates from HURH effluent were resistant to the above three antibiotics and Gentamycin. However *Shigella* isolates from YAH effluent were resistant to Tetracycline and Doxycycline, while only Tetracycline resistance observed for HURH effluent isolates. *E. coli* from both Hospital effluents was resistant to Ceftriaxone, Tetracycline, Gentamycin and Doxycycline. This similar pattern of resistance in both hospitals effluents may be due to similarity of type of antibiotic prescribed in both hospitals. However to determine the proportion of each species resistant to antimicrobials, large sample size for longer study period should be considered. To our knowledge, this study revealed the similarity in antimicrobial resistance pattern in both hospital effluents which warrants for appropriate interventions.

Recommendation

- As hospital effluents carry chemical and microbiological hazard, quality of effluent discharged in to receiving environment (water bodies) should be assessed in regular basis to minimize risk to public health.
- Activity of commonly used chemical disinfectants in hospitals should be tested to ensure minimal release of pathogenic bacteria in wastewater.
- Antibiotic susceptibility pattern of pathogenic and potentially pathogenic bacteria in hospital effluent should be regularly tested to reduce dissemination of resistant bacteria into the environment.
- Hospital wastewater generated should be treated by appropriate wastewater treatment plant before released into the environment.
- Large scale studies should be conducted in the region to reveal healthcare liquid waste management and microbiological quality of effluents discharged into receiving environment.

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Appendixes

AppendixI: Laboratory procedures

1.1 *Bacteriological characterization of wastewater*

Heterotrophic plate count, Procedure (spread plate method)

1. Prepare tenfold sample dilution in test tube
2. Prepare duplicate plates for each volume of sample or dilution examined
3. Pipette 0.5 mL sample using disposable syringe onto surface of predried tryptone glucose yeast agar plate.
4. Using a sterile bacteriological loop, distribute inoculum over surface of the medium by rotating the dish by hand.
5. Let inoculum be absorbed completely into the medium before incubating.
6. Incubate plate at $37^{O}C$ for 48 h.
7. Count number of colonies on plates having 30-300 colonies
8. Compute bacterial count per milliliter as follows;

$$CFU/mL= \frac{colonies\ \ counted}{Actual\ \ volume\ \ of\ \ sample\ \ in\ plate\ ,mL}$$

Staphylococcal count, Procedure (spread plate method)

1. Prepare tenfold sample dilution in test tube
2. Prepare duplicate plates for each volume of sample or dilution examined
3. Pipette 0.5 mL sample using disposable syringe onto surface of pre dried mannitol salt agar plate.

4. Using a sterile bacteriological loop, distribute inoculum over surface of the medium by rotating the dish by hand.
5. Let inoculum be absorbed completely into the medium before incubating.
6. Incubate plate at $35^{O}C$ for 48 h.
7. Count number of colonies on plates having 30-300 colonies
8. Compute bacterial count per milliliter as follows;

$$CFU/mL = \frac{colonies \ \ counted}{Actual \ \ volume \ \ of \ \ sample \ \ in \ plate \ ,mL}$$

Standard Total Coliform count, Procedure (most probable number or tube fermentation method)

1. Prepare Lauryl tryptose broth in double and single strength
2. Arrange 15 fermentation tubes in 3 rows (each row five tubes) in test tube rack
3. Dispense sufficient medium double strength in one row and single strength in second and third row of fermentation tubes with inverted Durham tubes
4. Sterilize in autoclave
5. Prepare tenfold dilution of the sample
6. Inoculate 1ml to each tube in a set of five with replicate sample volumes
7. Mix test portions in the medium by gentle agitation
8. Incubate inoculated tubes at 35 ± 0.5C.
9. After 24 ± 2 h swirl each tube gently and examine it for growth, gas, and acidic reaction (shades of yellow color) and, if no gas or acidic reaction is evident, reincubate and reexamine at the end of 48 ± 3 h.
10. Record presence or absence of growth, gas, and acid production in each tube
11. Count number of tubes giving positive reaction and calculate MPN as follows:

$$MPN/100mL = \frac{Number \ \ of \ \ positive \ \ tubes \ \ x100}{\sqrt{mL} \ sample \ \ in \ negative \ \ tubes \ \ x \ \sqrt{mL} \ sample \ \ in \ all \ tubes}$$

Fecal coliform count, Procedure (most probable number or tube fermentation method)

1. Prepare EC *broth in double and single strength*
2. Arrange 15 fermentation tubes in 3 rows (each row five tubes) in test tube rack
3. Dispense sufficient medium double strength *in one row and single strength in second and third row of fermentation tubes with inverted Durham tubes*
4. *Sterilize in autoclave*
5. *Prepare tenfold dilution of the sample*
6. Inoculate 1 ml to each tube in a set of five with replicate sample volumes
7. Mix test portions in the medium by gentle agitation
8. Incubate inoculated tubes at 44.5 ± 0.5^{0}C for 24 ± 2 h
9. Gas production with growth in an EC broth culture within 24 ± 2 h or less is considered a positive fecal coliform reaction
10. Count number of tubes giving positive reaction and calculate MPN as follows:

$$MPN/100mL = \frac{Number\ \ of\ \ positive\ \ tubes\ \ x100}{\sqrt{mL}\ sample\ \ in\ negative\ \ tubes\ \ x\ \sqrt{mL}\ sample\ \ in\ all\ tubes}$$

***E. coli* count,** Procedure (most probable number or tube fermentation method)

1. Prepare EC *–MUG broth in double and single strength*
2. Arrange 15 fermentation tubes in 3 rows (each row five tubes) in test tube rack
3. Dispense sufficient medium double strength *in one row and single strength in second and third row of fermentation tubes*
4. *Sterilize in autoclave*
5. *Prepare tenfold dilution of the sample*
6. Inoculate 1 ml to each tube in a set of five with replicate sample volumes
7. Mix test portions in the medium by gentle agitation
8. Incubate inoculated tubes at 44.5 ± 0.5^{0}C for 24 ± 2 h
9. Examine all tubes exhibiting growth for fluorescence using a long-wavelength (366 nm) UV light. The presence of bright blue fluorescence is considered a positive response for *E. coli*
10. Count number of tubes giving positive reaction and calculate MPN as follows

$$\text{MPN/100mL} = \frac{Number\ \ of\ \ positive\ \ tubes\ \ x100}{\sqrt{mL}\ sample\ \ in\ negative\ \ tubes\ \ x\ \sqrt{mL}\ sample\ \ in\ all\ tubes}$$

Enterococcal count, Procedure (most probable number or tube fermentation method)

1. Prepare brain heart infusion *broth in double and single strength*
2. Arrange 15 fermentation tubes in 3 rows (each row five tubes) in test tube rack
3. Dispense sufficient medium double strength *in one row and single strength in second and third row of fermentation tubes*
4. *Sterilize in autoclave*
5. *Prepare tenfold dilution of the sample*
6. Inoculate 1 ml to each tube in a set of five with replicate sample volumes
7. Mix test portions in the medium by gentle agitation
8. Incubate inoculated tubes at 44.5 ± 0.5^0C for 48 h
9. Examine all tubes exhibiting growth indicated by turbidity
10. Count number of tubes giving positive reaction and calculate MPN as follows:

$$\text{MPN/100mL} = \frac{Number\ \ of\ \ positive\ \ tubes\ \ x100}{\sqrt{mL}\ sample\ \ in\ negative\ \ tubes\ \ x\ \sqrt{mL}\ sample\ \ in\ all\ tubes}$$

Detection and isolation of pathogenic & potentially pathogenic bacteria

Salmonella spp

Procedure

1. Large volume (10 ml) of sample inoculated in selenite F broth and incubated at 37^oC for 48 h
2. Then streak from tubes with turbidity in to xylose lysine desoxycholate (XLD) agar and incubate at 37^OC for 24 h.
3. Suspected *Salmonella* spp produce black-centered pink colonies.
4. Pick suspect colonies then further identify by biochemical tests

Shigella spp

1. Large volume (10 ml) of sample inoculated in selenite F broth and incubated at 37^oC for 48 h
2. Then streak from tubes with turbidity in to xylose lysine desoxycholate (XLD) agar and incubate at 37^OC for 24 h.
3. Suspected *Shigella* spp produce pink colonies.
4. Pick suspect colonies then further identify by biochemical tests

S. aureus

Procedure

1. Inoculate sample on mannitol salt agar (MSA)
2. Pick mannitol fermenting yellow colonies & subculture on nutrient agar
3. Take colonies from nutrient agar and Perform Gram reaction (staphylococcus species are gram positive with grape morphology)
4. Perform slide catalase test using 3 % hydrogen peroxide (staphylococcus species are catalase positive)
5. Slide Coagulase test identify *S. aureus* from other species

E. coli (potentially pathogenic)

Procedure

1. Inoculate sample on MacConkey agar
2. Pick pink colonies and perform biochemical test

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 2 minutes.
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.

1.2 Determining disinfectant activity

1.2.1 Tincture iodine test

Procedure

1. Pipette 5-mL of sample in to sterile test tube
2. Add 5-mL of tincture iodine in to the sample tube
3. Mix by gentile shaking & put the tube for 5 min at room temperature
4. Streak plate 0.5 mL of the mixture on nutrient agar
5. Wait for some time until the water is absorbed on the agar plate
6. Incubate plate at $37^{O}C$ for 48 h
7. Observe colony of resistant bacteria if any colony present, further identify the bacteria
8. Take 3-4 pure colonies & inoculate in nutrient broth
9. Incubate overnight at $37^{o}C$ to have approximate bacterial population of $1 X 10^{6}/mL$

10. Prepare different concentration of disinfectant and treat with bacterial suspension. Then inoculate on nutrient agar. Use one plate as negative control
11. Determine MIC as logarithmic reduction;
    Log reduction = $\log_{10}$ CFU/mL (negative control) - $\log_{10}$CFU/mL (test). The highest log reduction considered as MIC.

1.2.2 Sodium hypochlorite test

Procedure

1. Prepare in-use dilution (1/10) of 5% sodium hypochlorite
2. Pipette 5-mL of sample in to sterile test tube
3. Add 5-mL of in-use sodium hypochlorite in to the sample tube
4. Mix by gentile shaking & put the tube for 5 min at room temperature
5. Streak plate 0.5 mL of treated sample on nutrient agar
6. Wait for some time until the water is absorbed on the agar plate
7. Incubate plate at 37^{O}C for 48 h
8. Observe colony of resistant bacteria if any present & further identify the bacteria
9. Take 3-4 pure colonies & inoculate in nutrient broth
10. Incubate overnight at 37^{o}C to have approximate bacterial population of $1 X 10^{6}$/mL
11. Prepare different concentration of disinfectant and treat with bacterial suspension. Then inoculate on nutrient agar. Use one plate as negative control
12. Determine MIC as logarithmic reduction
    Log reduction = $\log_{10}$ CFU/mL (negative control) - $\log_{10}$CFU/mL (test). The highest log reduction considered as MIC.

1.2.3 70% ethanol test

Procedure

1. Prepare in-use dilution 70% (v/v) ethanol
2. Pipette 5-mL of sample in to sterile test tube
3. Add 5-mL of 70 % ethanol in to the sample tube
4. Mix by gentile shaking & put the tube for 5 min at room temperature

5. Streak plate 0.5 mL of treated sample on nutrient agar
6. Wait for some time until the water is absorbed on the agar plate
7. Incubate plate at $37^{O}C$ for 48 h
8. Observe colony of resistant bacteria if any present & further identify the bacteria
9. Take 3-4 pure colonies & inoculate in nutrient broth
10. Incubate overnight at $37^{o}C$ to have approximate bacterial population of 1×10^{6}/mL
11. Prepare different concentration of ethanol and treat with bacterial suspension. Then inoculate on nutrient agar. Use one plate as negative control
12. Determine MIC as logarithmic reduction as follows

    Log reduction = $\log_{10}$ CFU/mL (negative control) - $\log_{10}$CFU/mL (test). The highest log reduction considered as MIC.

1.3 Antibiotic susceptibility testing

Method: disk diffusion

1. Using a sterile wire loop, touch 3–5 well-isolated colonies of similar appearance to the test organism and emulsify in 3–4 ml of sterile physiological saline or nutrient broth.
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 60^{O} 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.

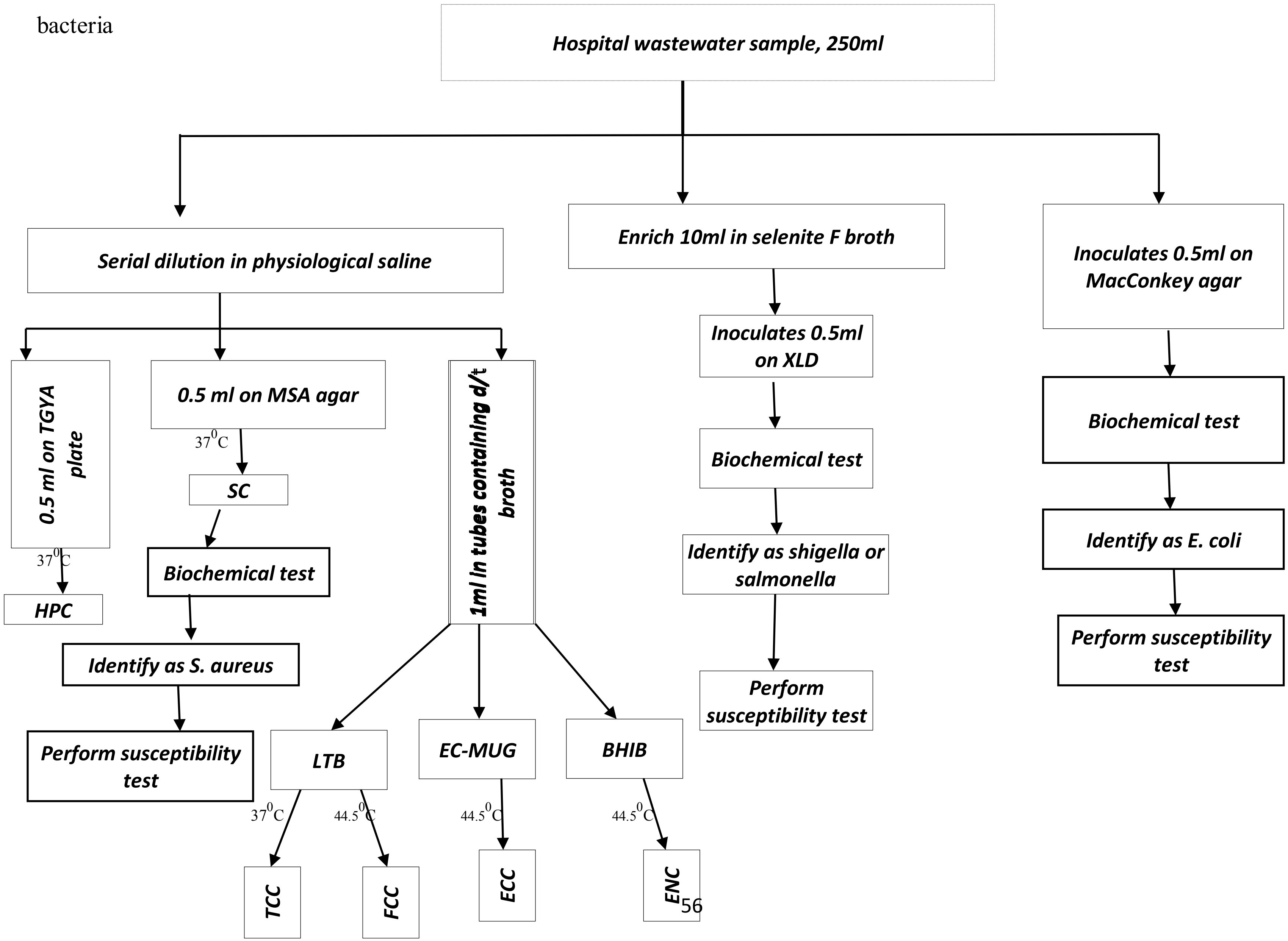
   Note: The discs should be about 15 mm from the edge of the plate and no closer than about 25 mm from disc to disc. No more than 6 discs should be applied (90 mm dish).

Each disc should be lightly pressed down to ensure its contact with the agar. It should not be moved once in place.

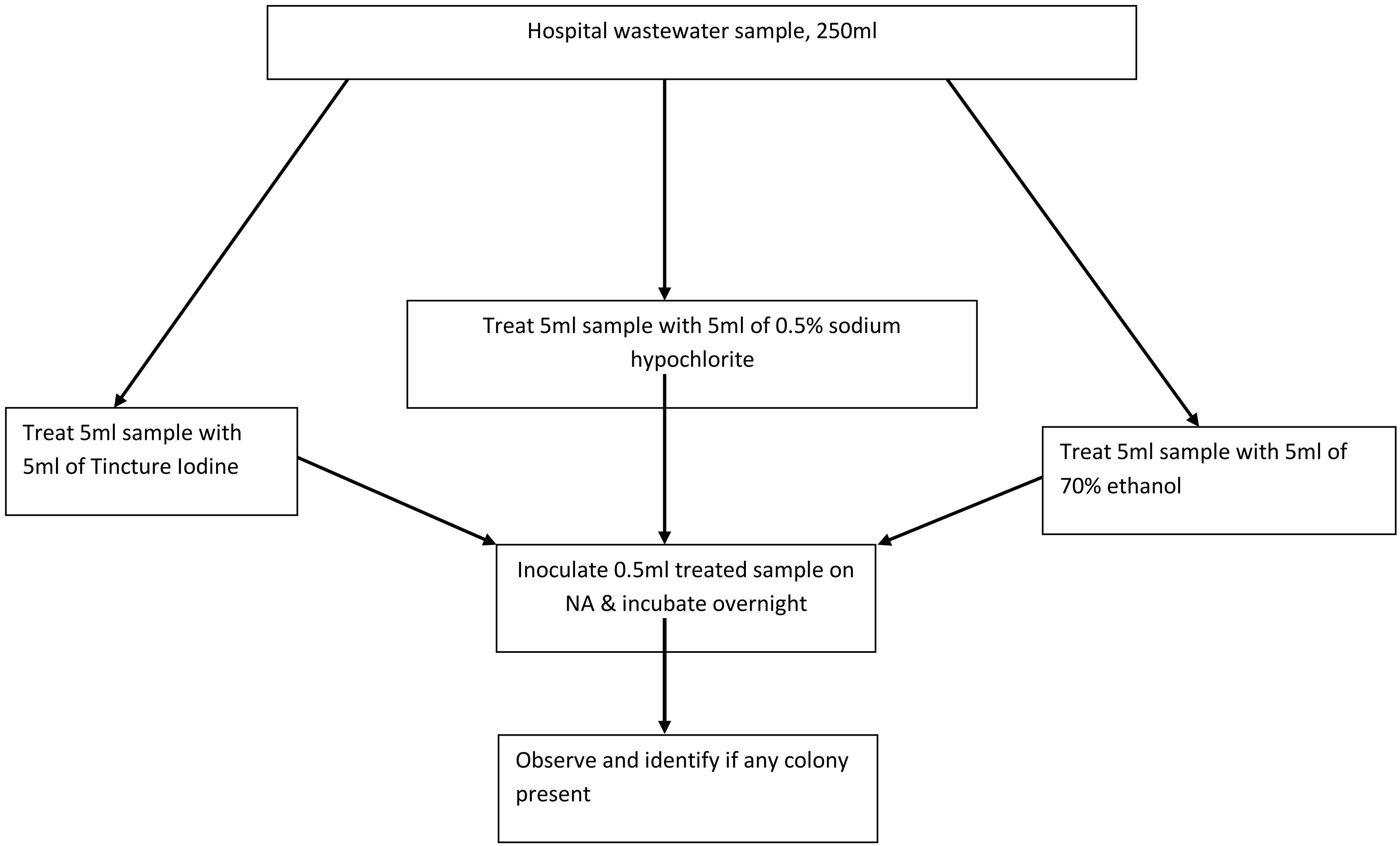
6. Within 30 minutes of applying the discs, invert the plate and incubate it aerobically at 35°C for 16–18 h (temperatures over 35°C invalidate results for oxacillin).
7. After overnight incubation, examine the control and test plates to ensure the growth is confluent or near confluent. Using a ruler 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.

Appendix II: Procedure flow chart

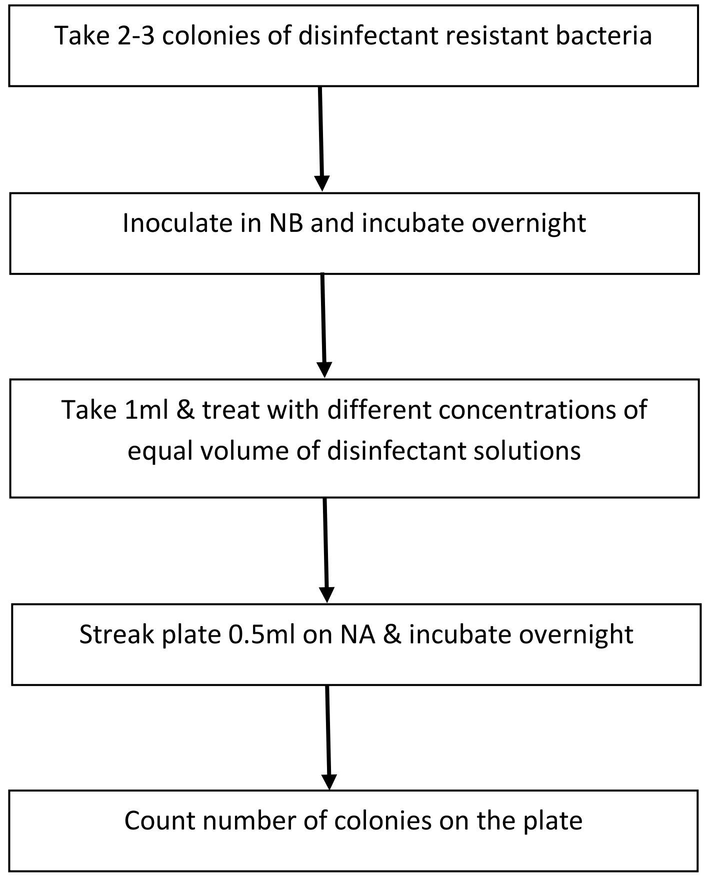
2.1 Flow chart showing procedure for determination of indicator organisms, identification & susceptibility testing for pathogenic bacteria



2.2 Flow chart for evaluation of disinfectant activities



2.3 Flow chart for MIC determination



Appendix III: Data collection format

3.1 Hospital wastewater sample collection format

Date of collection: ________________________

Hospital site: ____________________________

Sample number: __________________________

Collection time: __________________________

Sampled by: _____________________________

Signature: _______________________________

3.2 Bacteriological analysis and laboratory result registration format for total heterotrophic count

Investigator:

Name: ________________________________
Signature: _____________________________
Date: __________________________________

Sample number	Hospital site	Total heterotrophic count (CFU/mL)	Comment
	YH		
	YH		
	YH		
	YH		
Average YH			
	HURH		
	HURH		
	HURH		
	HURH		
Average HURH			

Note: YH-Yirgalem hospital, HURH-Hawassa University Referral Hospital

3.3 Bacteriological analysis and laboratory result registration format for total coliform , *E. coli* & Enterococci count

Investigator:

Name: ________________________________

Signature: ______________________________

Date: __________________________________

Sample number	Hospital site	Total coliform , *E. coli* & Enterococci count (CFU/mL)			Comment
		Total coliform	*E. coli*	*Enterococci*	
	YH				
	YH				
	YH				
	YH				
Average YH					
	HURH				
	HURH				
	HURH				
	HURH				
Average HURH					

Note: YH-Yirgalem Hospital, HURH-Hawassa University Referral Hospital

3.4 Bacteriological analysis and laboratory result registration format for *Pseudomonas and staphylococcus count*

Investigator:

Name: ________________________________

Signature: ______________________________

Date: __________________________________

Sample number	Hospital site	Bacterial count (CFU/mL)		Comment
		Pseudomonas	*Staphylococcus*	
	YH			
	YH			
	YH			
	YH			
Average YH				
	HURH			
	HURH			
	HURH			
	HURH			
Average HURH				

Note: YH-Yirgalem Hospital, HURH-Hawassa University Referral Hospital

3.5 Bacteriological analysis and laboratory result registration format for common pathogenic bacterial isolates from Hospital wastewater

Investigator:

Name: ______________________________

Signature: ____________________________

Date: ________________________________

S.No	Hospital site				Comment
	YH		HURH		
	GN	GP	GN	GP	

Note: YH-Yirgalem Hospital, HURH-Hawassa University Referral Hospital, GN-Gram negative, GP-Gram positive

3.6 Bacteriological analysis and laboratory result registration format for MIC determination by agar dilution method for resistant bacterial species

Investigator:

Name: ________________________________

Signature: ______________________________

Date: __________________________________

Hospital site	Resistant bacteria isolated and tested	Disinfectant used and Its MIC			Comment
YH		Alcohol (ethanol)	Provipone Iodine	Sodium hypochlorite	

Investigator:

Name: ________________________________

Signature: ______________________________

Date: __________________________________

Hospital site	Resistant bacteria isolated & tested	Disinfectant used and Its MIC			Comment
HURH		Alcohol (ethanol)	Providone Iodine	Sodium hypochlorite	

3.7 Bacteriological analysis and Laboratory result registration format for susceptibility testing by disk diffusion method for selected antibiotics

Investigator:

Name: ______________________________

Signature: ____________________________

Date: ________________________________

S.No	Organism tested	Antibiotic :________________		Comment
		Susceptible (S)	Resistant (R)	

Assurance of the Principal Investigator

The undersigned agrees to accept responsibility for the scientific, ethical and technical conduct of the research project and for the provision of required progress reports as per terms and conditions of the research publications office in effect at the time of grant is forwarded as the result of this publication.

Name of the student: Sintayehu Fekadu, BSc (M.Sc candidate)

Signature-------------------

Date and place of submission------------------

Addis Ababa, Ethiopia

Assurance of the Advisor

Name of the advisor: Dr. Solomon Gebre-Selassie, MD, M.Sc

Signature-------------------

Date and place--------------------

Addis Ababa, Ethiopia