Original Article

Assessment of antibiotic- and disinfectant-resistant bacteria in hospital wastewater, south Ethiopia: a cross-sectional study

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Abstract

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

Methodology: A cross-sectional study was conducted between December 2010 and February 2011 on hospital wastewater. A total of 24 composite samples were collected on a weekly basis for bacteriological analysis and susceptibility testing. Indicator organisms and pathogenic and potentially pathogenic bacteria were found and isolated on selective bacteriologic media. Disinfectant activity was evaluated by use-dilution, and minimum inhibitory concentration (MIC) was determined by the agar dilution method. Similarly, antibiotic susceptibility tests were performed using the Kirby-Bauer disk diffusion method.

Results: Pathogenic (*Salmonella*, *Shigella*, and *S. aureus*) and potentially pathogenic (*E. coli*) bacteria were detected from effluents of both hospitals. Dilution demonstrated tincture iodine to be the most effective agent, followed by sodium hypochlorite; the least active was 70% ethanol. MIC for ethanol against *S. aureus* and Gram-negative rods from Yirgalem Hospital (YAH) showed 4 and 3.5 log reduction, respectively. *Salmonella* isolates from YAH effluent were resistant to ceftriaxone, tetracycline, and doxycycline. Isolates from Hawassa University Referral Hospital (HURH) effluent were resistant to the above three antibiotics as well as gentamycin.

Conclusions: Hospital effluents tested contained antibiotic-resistant bacteria, which are released into receiving water bodies, resulting in a threat to public health.

Key words: antibiotic; disinfectant; hospital effluent; hospital influent.

Introduction

Wastewater refers to any water whose quality has been compromised by human activities. It includes liquid waste discharged from domestic homes, agricultural commercial sectors, pharmaceutical sectors, and hospitals. In hospitals, water is consumed by various areas such as hospitalization rooms, surgery rooms, laboratories, administrative units, laundries, and kitchens. In the process, its physical, chemical, and biological quality is decreased and converted to wastewater [3]. A variety of substances, such as pharmaceuticals, radionuclides, antiseptics, disinfectants, and solvents are used in hospitals for treatment, medical diagnostics, disinfection, and research. Many non-metabolized drugs excreted from patients and residual chemicals enter into wastewater, which finally interacts with the microflora of hospital sewage. These microflora comprise saprophytic bacteria from the atmosphere, soil, medical devices, and water employed in the hospital practice; the pathogens are mainly released with patient excreta. These bacteria are 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 does domestic wastewater [4].

The public health impact of the release of resistant bacteria to the receiving environment involves a number of points. First, if the resistant bacteria are carrying a transmissible gene, they transfer resistant genes to other community bacteria so that infection caused by these bacteria are usually difficult to treat, and it also decreases the antibiotic pool for the treatment of bacterial infections. 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 the costs of treatment and hospitalization [4].

The current study was designed to assess the pattern of antimicrobial resistance of bacteria isolated from the effluent of two major hospitals against commonly used antimicrobial agents. The findings of this study will help to make public health authorities aware of the dissemination of resistant bacteria in the receiving water bodies and will provide information about the proper management of hospital effluents.

Methodology

Study design

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

Study area and period

The study was carried out in Yirgalem Hospital and Hawassa University Referral Hospital, the two big hospitals in south Ethiopia.

Yirgalem Hospital (YAH) operates with 200 licensed beds and uses all available beds for patient management. The hospital releases approximately 125 cubic meters of partially treated (pretreatment septic tank) effluent per day to an open field. The hospital has a previously used physical wastewater treatment plant (sand filter) that is now non-functional.

Hawassa University Referral Hospital (HURH) is a teaching hospital established in 2005. The hospital is located near Lake Hawassa and operates with 350 licensed beds. The wastewater treatment system used is an oxidation pond system which comprises two facultative ponds and two maturation ponds for the treatment of the wastewater, and an additional fish pond for fish farming. The facultative ponds receive a combination of settled wastewater from a septic tank pre-treatment and raw wastewater from the student dormitory and staff residence buildings. The last step in treatment process is the release of approximately 143.3 cubic meters of treated effluent per day, which joins Lake Hawassa after flowing through approximately 20 meters of the sewage system.

Sample collection

A total of 24 composite hospital wastewater samples were collected from HURH (eight influent and eight effluent samples), and eight effluent samples were collected from YAH on a weekly basis between December 2010 and February 2011. Each partial sample was collected at 8:30 a.m., 10:30 a.m., 12:30 a.m., and 2:30 a.m. in a small sterile bottle according

to the method used by Nuñez and Moretton [5]. The samples were transferred into 250 mL-sized sterile bottles containing 0.2 mL of 3% w/v sodium thiosulphate and then transported within two hours in ice jackets in an ice box to the microbiology laboratory for analysis and stored in a refrigerator at 4°C until analysis. All the samples were analyzed on the day they were collected.

Bacteriological enumeration of wastewater

To determine the total heterotrophic plate count, serial 10-fold dilutions of samples were prepared in physiological saline, and 0.5 mL aliquot was streak plated on tryptone glucose yeast agar (TGYA). Plates were incubated for 48 hours at 37ºC before bacteriological counts were done. The number of colonies on duplicate plates having 30–300 colonies was counted by using a digital colony counter. Finally, the bacterial count was reported CFU/mL as follows:

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

For the *Staphylococcus* count, appropriate dilutions were prepared and 0.5 mL of aliquot was streaked on mannitol salt agar (MSA) and incubated at 37°C for 24 to 48 hours. Colonies showing a typical yellow zone of fermentation were used for Gram staining. Those colonies identified as Gram-positive cocci were counted using a digital colony counter as staphylococci according to the method used by Dudely *et al.* [6]. Reporting similar to that described above was applied to determine the CFU/mL.

For the total coliform count, serial 10-fold dilutions of samples were prepared in physiological saline, and 1 mL of aliquot was transferred aseptically into a series of test tubes containing Durham tube and lauryl tryptose broth (LTB). Tubes were gently shaken and incubated for 48 hours at 37ºC. Production of gas and lactose fermentation were observed as positive reactions.

For the fecal coliform count, serial 10-fold dilutions of sample were prepared in physiological saline and 1 mL of aliquot was transferred aseptically into a series of test tubes containing Durham tube and lauryl tryptose broth. Tubes were gently shaken and incubated for 48 hours at 44.5ºC. Production of gas and lactose fermentation were observed as positive reactions.

For the *Escherichia coli* count, serial 10-fold dilutions of sample were prepared in physiological saline and 1 mL of aliquot was transferred aseptically

into a series of test tubes containing *Escherichia coli* 4-methylumbelliferyl-β-glucuronide (EC-MUG) medium. Tubes were gently shaken and incubated for 48 hours at 44.5ºC, then all tubes were examined for growth of bright blue fluorescence using a long wavelength UV lamp, which was considered a positive response for *E. coli*.

For the enterococci count, Serial 10-fold dilution of sample was prepared in physiological saline and 1 mL of aliquot was transferred aseptically into a series of test tubes containing brain-heart infusion broth (BHIB). Tubes were gently shaken and incubated for 48 hours at 44.5ºC, then all tubes were examined for turbidity and considered as positive if turbid.

All methods were used according to standard methods for examination of water and wastewater developed by American Public Health Association (APHA) [7].

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

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

The most important pathogenic bacteria found in hospital wastewater were identified based on their colony appearance, Gram staining, growth on selective media, and biochemical tests according to the standard methods for examination of water and wastewater developed by APHA [7].

Disinfectant susceptibility testing
To test the effectiveness of tincture iodine, 5 mL of effluent sample was treated with 0.1% of 5 mL tincture iodine for 5 minutes in a sterile test tube. Then, 0.5 mL of aliquot was streak plated on nutrient agar and incubated for 48h at 37ºC.

To test the effectiveness of sodium hypochlorite, 5 mL of effluent sample was treated with 0.5% of 5 mL sodium hypochlorite for 5 minutes in a sterile test tube. Then, 0.5 mL of aliquot was streak plated on nutrient agar and incubated for 48 hours at 37ºC.

To test the effectiveness of 70% ethanol (ethyl alcohol), 5 mL of effluent sample was treated with 70% of 5 mL ethanol for 5 minutes in a sterile test tube. Then, 0.5 mL of aliquot was streak plated on nutrient agar and incubated for 48 hours at 37ºC.

Finally, the growth of colonies was observed and if any were present, the bacteria were identified and minimum inhibitory concentration (MIC) was

determined according to methods suggested by Hani and Adnan [8].

Minimum inhibitory concentration
Minimum inhibitory concentration (MIC) for 70% ethanol resistant organisms was determined as follows:MIC values of ethanol were determined on tubes containing 1 mL of 60%, 65%, 70%, 75%, and 80% of ethanol. One mL of bacterial suspension with a concentration of approximately 1×10^6/mL were transferred into each tube and treated with different concentration of ethanol for 5 minutes. Then nutrient agar plates were inoculated with 0.5 mL of treated suspension. All plates were incubated for 48 hours at 37ºC and the number of colonies was counted. The MIC was the lowest concentration that prevented bacterial growth [9]. Bactericide activities were expressed as reduction factors, that is, logarithmic reductions in viable organisms: reduction factor ($\log_{10}$ CFU/mL $_{(negative\ control)}$ - $\log_{10}$ CFU/mL $_{(treated)}$) was determined according to Hani and Adnan [8].

Antibiotic susceptibility testing
The following antibiotics are commonly prescribed in the two hospitals: ceftriaxone, ciprofloxacin, ampicillin, gentamycin, doxycycline, amoxicillin, tetracycline, vancomycin, and penicillin. The susceptibility of pathogenic and potentially pathogenic bacteria isolates for these antibiotics was determined using the Kirby-Bauer disk diffusion method [9]. All the antibiotic disks used were from Oxoid Company (Basingstoke, UK). A sterile swab was dipped in a bacterial suspension (McFarland standard 0.5, with approximate bacterial population of 1×10^6 CFU/mL) and streaked onto Müeller-Hinton Agar. Antibiotic disks were applied using a sterile forceps. Agar plates were incubated at 37°C for 18 hours, and the zone of inhibition was measured in millimeters using a ruler. Interpretation was made using susceptibility breakpoints annually published by the National Committee for Clinical and Laboratory Standards Institute (CLSI) [9].

Quality control
The quality of media, reagents, stains, antibiotic disks, and disinfectant solutions were insured following the manufacturer's instructions. In addition, reference strains were obtained from the Ethiopian Health and Nutrition Research Institute (EHNRI) and employed to check the performance of disk diffusion tests and biochemical tests: *Pseudomonas aeruginosa* (27853), *Escherichia coli* (25922) and *Staphylococcus*

aureus (25923) according to CLSI recommendations [10].

Data analysis

Data were entered, cleaned, and analyzed using SPSS version 16.0. Descriptive statistics were employed to report numerical summaries of findings.

Ethical considerations

This work was approved by the Research and Ethical Committee of the Department of Microbiology, Immunology and Parasitology, School of Medicine, Addis Ababa University. Findings obtained were communicated to the respective hospitals for better management of hospital effluents.

Results

Enumeration of indicators and identification of pathogenic organisms

Table 1 shows the average number of indicator bacteria in sampling sites and shows that HURH influent had the highest number of bacteria compared to HURH and YAH effluent.

A total of 16 samples, 8 influent and 8 effluent samples, were tested for indicator organisms before and after treatment of wastewater in HURH. The result shown in Table 2 indicate the average percent reduction of organisms by wastewater treatment plant (oxidation pond).

The study also revealed the presence of a 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 such as *S. aureus*, *Salmonella*, and *Shigella* from wastewater of both hospitals were identified. A total of 19 *Salmonella* (7 from YAH effluents, 7 from HURH influents, and 5 from HURH effluents) and 12 *Shigella* (4 from YAH effluents, 5 from HURH influents, and 3 from HURH effluents) were detected.

The rate of detection and identification of *Salmonella* was higher compared to that of *Shigella* at all sites. Although the rate of reduction of pathogenic bacteria was much lower as compared to indicator organisms, the highest reduction was observed in *E. coli* followed by *Shigella* (Table 3).

Disinfectant and antibiotic susceptibility

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 to 70% ethanol, which was the least effective disinfectant.

The results of MIC of ethanol showed that the inhibitor effect or germ-killing power of ethanol was significantly reduced at 60% and 80%. The optimum concentration that 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.

Table 1. Geometric mean of indicator organism in HURH & YAH wastewater, 2010/2011

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

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

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
Staphylococcalcount 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

Table 4. 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 5. Antibiotic susceptibility pattern of *Salmonella*, *Shigella*, and *E. coli* isolated from effluents of HURH, 2010/2011

Antibioticconcentration♠	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 6. Antibiotic susceptibility pattern of *S.aureus* isolated from YAH and HURH effluents, 2010/2011

Antibioticconcentration♠	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

Similarly, the optimum concentration that 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.

Salmonella isolates from YAH effluent were resistant to ceftriaxone, tetracycline, and doxycycline, whereas those from HURH effluent were resistant to the above three antibiotics and gentamycin as well. Patterns of susceptibility to different antibiotics are shown in Tables 5, 6, and 7.

Discussion

In this study, some pathogenic and potentially pathogenic bacteria were detected. *S. aureus* and *E. coli* were detected in high concentration from the effluents of both hospitals, as indicated in Table 1. There were differences in the detection of *Salmonella* and *Shigella* in both hospitals. Dudley *et al.* [6] reported a variety of pathogenic and potentially pathogenic bacteria in sewage sludge, with *Shigella* not detected due to low sensitivity of the enrichment procedure and the high temperature that decreased its survival. The high frequency of detection of pathogenic bacteria in our study may be due to the admission of cases with these bacterial infections, which are common in developing countries like Ethiopia. Although the number of samples in this study was small, lesser detection of *Salmonella* and *Shigella* in HURH effluent may be due to reduction through the treatment process. Similarly, Momba *et al.* [11] reported gradual removal of presumptive bacterial pathogens in different zones of treatment plants. In their findings, there were variation with regards to both the patterns and efficiency of each plant for the removal of the target pathogens. 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*.

Staphylococcus aureus was detected in high numbers in all effluent samples from both hospitals and was continuously released to the receiving environments. This is in line with other studies that reported that the organism is resistant to antiseptics, disinfectants, and antibiotics and survives in the sewage system for long periods of time [5,12,13]. Contamination of rivers and lakes with this pathogen may pose a risk to public health associated with staphylococcal infection and food poisoning.

In our study, pathogenic bacteria were detected in the wastewater treatment facility in HURH. This may be due to the inefficient removal of pathogenic bacteria by the treatment process or due to the admission of large numbers of cases with these bacterial infections that were subsequently released to the wastewater.

Although bacterial resistance to antibiotics has been extensively studied in both clinical and environmental samples, only a few reports are available on disinfectant activity against microorganisms.

Iodine has long been considered an effective antimicrobial agent, especially when used in the form of providone-iodine [5]. This study revealed that tincture iodine-treated effluent showed no growth on nutrient agar after incubation. This finding is in agreement with those of Favero and Drake [14], which found iodine to be an efficient microbicide compared to chlorine in swimming pools. However, Nuñez and Moretton [5] reported iodine-resistant organisms such as *S. epidermidis*, *Bacillus* spp. and *Pseudomonas alcaligenes* and reported that resistance increased in a nutrient-restricted environment such as the oligotropic aquatic system found in hospital waste water (HWW).

In effluent treated with sodium hypochlorite (with 0.5% free chlorine), *Bacillus* spp. was resistant and grown in plate. Similar observations were reported in different studies [15,16]. However, a comparative study conducted in Brazil to compare disinfectant activity against a standard strain, antibiotic susceptible and resistant hospital strains revealed that sodium hypochlorite was effective against all strains tested [13].

Antibiotic susceptibility test results for penicillin, ampicillin, tetracycline, vancomycin, gentamycin, ciprofloxacin, erythromycin, doxycycline, and ceftriaxone against important organisms are indicted in Tables 4, 5, and 6. Direct comparison of our findings with those of other scholars was difficult because of differences in types of antibiotics used, types of wastewater samples collected, and types of organisms isolated. The susceptibility pattern (Tables 4 and 5) against *E. coli* revealed that the organism was resistant to ceftriaxone, tetracycline, gentamycin, and doxycycline. A similar study conducted in Bangladesh showed that all *E. coli* isolates from untreated HWW were multidrug resistant ($\geq$ 4 antibiotics), resistant to tetracycline (100%), ciprofloxacin (100%), penicillin (100%), erythromycin (100%), gentamycin (50%), and chloramphenicol (90%), and that all of them were sensitive to imipenem.

Our result (Tables 4 and 5) showed that isolates from YAH effluents were resistant to ceftriaxone, tetracycline, and doxycycline, whereas isolates from

HURH effluents were resistant to the above three antibiotics and gentamycin. A study conducted on municipal water treatment plants in two California cities showed that a set of *Salmonella* serovars were resistant to multiple antibiotics, with many serovars sharing the resistant phenotypes, and that there was no significant difference in the levels of multiple antibiotic resistance between the two study sites [3]. The antibiotic susceptibility pattern of *S. aureus* is well studied in clinical samples; however, very limited reports are available on resistance patterns of isolates from hospital effluents. The susceptibility pattern to common antibiotics (Table 6) 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.

This study had some limitations. First, the wastewater sample was taken over a short period and may not have depicted seasonal variation. Second, because only wastewater was used, the study was unable to differentiate the source of resistant bacteria (clinical isolates or sewage system).

Conclusions

Wastewater from both hospitals contained pathogenic (*Salmonella* spp., *Shigella* spp., and *S. aureus*) and potentially pathogenic (*E. coli*) bacteria. There were differences in the detection of pathogenic bacteria in both hospitals, with higher frequency in the YAH effluent; YAH requires proper treatment of wastewater before releasing it into the receiving environment. Also, this study revealed that the two hospital effluents tested contained antibiotic-resistant bacteria that were released to receiving water bodies, consequently posing a public health threat.

Authors' contributions
Sintayehu Fekadu has developed research proposal, collected sample, carried out laboratory investigations, and wrote research paper. Dr. Solomon Gebersillase was the protocol advisor during proposal development and actual research. Yared Merid was co-advisor at different level of the research project. Hunachew Beyene was co-advisor at different levels of the research. Wondu Teshome carried out data entry and statistical analysis.

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