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Antibiotic Resistant Bacteria from Treated and Untreated Hospital Wastewater at Ayder Referral Hospital, Mekelle, North Ethiopia

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Abstract

The widespread emergence of antibiotic resistance among bacterial pathogens has become one of the most serious challenges in Ethiopia. This study determined the prevalence and drug resistance patterns of bacterial pathogens isolated from treated and untreated wastewater released from Ayder Referral Hospital in Northern Ethiopia. A cross sectional study design was conducted from September-December, 2015 in wastewater released from Ayder referral hospital. A total of 40 composite samples were aseptically collected, transported and processed for enumeration of indicator organisms, bacteriological identification and susceptibility testing following standard procedure. Data obtained were entered and analyzed using SPSS version 20. Mean heterotrophic plate count, total coliform count, fecal coliform count and *E. coli* count were found to be 1.6×10^6 CFU/mL, 2.2×10^6 CFU/100 mL, 2.0×10^5 CFU/100 mL and 1.1×10^4 CFU/100 mL from treated wastewater respectively. Among the total samples 134 bacterial isolates were detected and [84 (62.7%)] were from untreated wastewater and [50 (37.3%)] were from treated wastewater. The most frequently isolated bacteria from untreated wastewater samples was *Klebsiella* spp [14 (16.7%)] followed by *S. aureus* [13 (15.5%)] and *P. aeruginosa* [12 (14.3%)], similarly in treated wastewater samples *Klebsiella* spp [10 (20%)], *P. aeruginosa* [8 (16%)] and *S. aureus* [8 (16%)] were frequently detected. The overall multi-drug resistance (MDR) in this study was [79/134 (79.1%)]. MDR from untreated wastewater sample was [64/84 (76.2%)] while from treated wastewater sample was [42/50 (84%)] and shows significant difference with (COR: 1.64, 95% CI: 1.15 - 3.29, P: 0.001). It is concluded that treated hospital wastewater contains large numbers of antibiotic

resistant bacteria. Therefore, there should be continuous monitoring and evaluation of the effluent quality of the ponds and chlorination of the final effluent should be developed.

Keywords

Indicator Organism, Bacterial Isolates, Drug Resistance,
Treated Wastewater, Untreated Wastewater

1. Introduction

Wastewater is an ideal media for a wide range of microorganisms especially bacteria, viruses and protozoa, and used as a reservoir for resistant bacteria [1]. It carries the resistant bacteria introduced into the sewage system that come from human excretions, liquid waste discharged from domestic home, agricultural and commercial sectors, pharmaceutical and hospitals [2] [3].

The volume of antibiotics used in hospitals released into effluent results a selection pressure on bacteria. Therefore, wastewater from hospitals contains high numbers of resistant bacteria and antibiotic residues at concentrations able to inhibit the growth of susceptible bacteria [4]. Accordingly, hospital wastewater could increase the numbers of resistant bacteria in the recipient sewers by both mechanisms of introduction and selection pressure [5]. The release of resistant bacteria to the receiving environment can pose public health impact through, carrying transmissible gene, by acting as a vector or reservoir of resistant gene [6] [7]. The most common bacterial pathogens found in hospital wastewater are *Salmonella*, *Shigella*, *S. aureus*, *P. aeruginosa*, *Vibrio*, *Clostridium*, *Yersinia, Campylobacter*, *Leptospira* and groups of total coliforms consisting of *Serratia, Enterobacter*, *Klebsiella*, and *Escherichia coli* (*E. coli*) [8] [9]. Those bacterial pathogens are among of the most dangerous contaminants on human health, and then the effluents from hospital wastewaters are one of the most serious pollutants discharging to the environment [9] [10]. The multidrug resistance patterns seen in the bacterial isolates from hospital effluent samples include most of the antibiotics used presently for treating human infections. The worst fear apprehended is the transfer of such resistance to bacterial pathogens causing infections in the community. In that case most of the presently available antibiotics will be vain against the infectious organisms. The origin of such multidrug resistance bacterial strains appears to be the hospital environment due to selective pressure [11]. Dissemination of antimicrobial resistance bacteria in the environment is a major problem in developing countries, mainly due to improper antibiotic usage, ineffective infection control program and lack of better management of hospital wastewater. In Ethiopia, rapid urbanization and industrialization with improper environmental planning often lead to discharge of industrial and hospital sewage effluent directly to the environment also a problem [12] [13].

2. Materials and Methods

2.1. Study Area

Ayder referral hospital has wastewater stabilization pond as treatment system. The treatment plant consists of seven rectangular slanty ponds. The ponds are lined at the bottom by thick plastic to minimize seepage into ground water zones. The first pond (facultative pond) is the largest of all, having greater length and width from the other ponds and receives raw wastewater. It is primarily designed for BOD removal and has three zones in vertical section; those are supper aerobic zone, middle facultative zone or intermediate zone and lower anaerobic zone. Maturation ponds consist of six slanty ponds having almost similar length and width and have shallow depth. It receives wastewater effluent from facultative pond and its primary function is removal of pathogenic microorganisms. The last step in treatment process is release of treated effluent to open field continuously after flowing approximately 50 meter sewage system. The Untreated Hospital Wastewater (UHWW) sampling site was just the inlet of treatment plant while the Treated hospital Wastewater (THWW) sampling site was just at the outlet of treatment plant before released to the open field.

2.2. Study Design and Period

Cross sectional study design was conducted to collect hospital wastewater samples from September, 2015 to December, 2015.

2.3. Sampling Frequency and Sampling Technique

A "Composite-sampling" technique was applied to collect the most representative samples according to guidelines of wastewater sampling techniques stated on APHA [14]. A total of 80 partial samples were collected from both sampling site with 4 hour interval in the study period and finally 40 composite samples were processed for bacteriological analysis, identification and susceptibility testing. Partial samples were collected two times a day with four hour interval from both sites in 125 mL cleaned and sterile microbiological glass bottles. The first samples were collected in the morning 10 AM from UHWW sampling site and 10:30 AM from THWW sampling site and transported immediately after collection and stored in refrigerator at 4˚C. After collection of afternoon samples 2 PM from UHWW and 2:30 PM from THWW, the partial samples were pooled (untreated to untreated and treated to treated) aseptically to 250 mL sized cleaned and sterilized microbiological glass bottle. Then it was stored in refrigerator at 4˚C until the samples is processed.

2.4. Bacteriological Analysis of Wastewater

Total heterotrophic plate count: Duplicate plates were prepared for each volume of sample examined. All the samples were vigorously shaken before preparation of dilutions then serial 10-fold dilutions of samples were prepared in physiological saline, and 1 mL aliquot was spread over nutrient agar plate (Himedia).

Then plates were incubated for 48 h at 37˚C before bacteriological counts were performed. Number of colonies on duplicate plate having 30 - 300 colonies was counted and finally bacterial count was reported as 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 a series of 9 test tubes containing Durham tubes and double strength MacConkey broth (Himedia). Tubes were gently shaken and incubated for 24 - 48 h at 37˚C, then production of gas and lactose fermentation was taken as positive reaction. Finally bacterial load were estimated using MPN table and dilution factor. The bacterial count was reported as CFU/100 mL.

Fecal coliform count: Serial 10-fold dilutions of sample were prepared in physiological saline and 1mL of aliquot was transferred aseptically in to a series of 9 test tubes containing Durham tubes and double strength MacConkey broth. Tubes were gently shaken and incubated for 24 h at 44.5˚C in water bath, then production of gas and lactose fermentation was taken as positive reaction. Furthermore *E. coli* cont was confirmed by adding approximately 0.1 ml of Kovacs reagent (Uni-Chem). Finally bacterial load were estimated using MPN table and dilution factor. The bacterial count was reported as CFU/100 mL.

2.5. Isolation and Identification of Bacteria

Culture technique: The sample was investigated for further isolation and identification of bacterial pathogens. Representative diluted sample of 1 ml aliquots was plated on selective and differential media. MacConkey agar (SRL-sisco research laboratory), Salmonella-shigella agar (Uni-Chem) and Mannitol salt agar (Himedia) were used and prepared according to manufacturer direction. After obtaining pure colonies and recording important features of the isolated organisms, further identification were done using gram staining and biochemical test with standard methods.

Biochemical test: Gram negative bacteria was identified based on colonial morphology and pigmentation, Oxidase test, Carbohydrate fermentation, H_2S production, Citrate utilization, motility, growth at 42˚C, Indole formation, Lysine decarboxylase and Lysine deaminase production, and Urea hydrolysis. Gram positive isolates were also differentiated by gram staining, colonial characteristics, catalase test and coagulase tests.

2.6. Antimicrobial Susceptibility Testing

A standard Kirby-Bauer disk diffusion method was used to determine the antimicrobial susceptibility profiles of the isolates as described by the CLSI, 2014 [15]. Bacterial isolates were tested for the following commonly prescribed drug: Erythromycin (15 μg), Gentamyacin (10 μg), Amikacin (30 μg), Amoxicillin-Clavulunic acid (30 μg), Ceftriaxone (30 μg), Ciprofloxacin (5 μg), Tetracycline (30 μg), Cotrimoxazole (25 μg), Ampicillin (10 μg), Penicillin (10 μg), Chloromaphenicol (30 μg), Doxycycline (30 μg) and Cefoxitin (30 μg). Interpretation was made using susceptibility breakpoints of CLSI, 2014 and diameter of

the zone of inhibition around the disc was measured to the nearest millimeter using a metal caliper and the isolates were classified as sensitive, intermediate and resistant.

2.7. Data Quality Assurance

Sample collection, handling, transportation and microbiological analysis and interpretation of results were carried out using standard operating procedures (SOPs). Prior to the actual work Reagents, media and antimicrobial disks were checked for expiry date, damage and storage problems. Laboratory equipment's were properly cleaned and sterilized before use. Media preparation was made based on the respective manufacturer's directions. 5% of media per batch/prepared was incubated overnight for sterility check. *Escherichia coli* (ATCC 25922), *Staphylococcus aureus (*ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 28753) were used as quality control organisms.

2.8. Data Management and Statistical Analysis

Data was entered and summarized using SPSS version 20 software (USA) and analyzed using the STATA software (StataCorp LP, College. Station, Texas, USA). A *p*-value of ≤0.05 was considered indicative of a statistically significant difference.

3. Results

3.1. Bacteriological Analysis of Wastewater before and after Treatment

Mean heterotrophic plate count, total coliform count, fecal coliform count and *E. coli* count were found to be 1.6×10^6 CFU/mL, 2.2×10^6 CFU/100 mL, 2.0×10^5 CFU/100 mL and 1.1×10^4 CFU/100 mL from THWW respectively (Table 1).

3.2. Bacterial Isolates

Among the total samples 134 bacterial isolates were detected and 84 (62.7%) were from UHWW and 50 (37.3%) were from THWW. The most frequently isolated bacteria from untreated wastewater samples was *Klebsiella* spp 14 (16.7%) followed by S. *aureus* 13 (15.5%) and *P. aeruginosa* 12 (14.3%), similarly in treated wastewater samples *Klebsiella* spp 10 (20%), *P. aeruginosa* 8 (16%) and *S. aureus* 8 (16%) were frequently isolated (Table 2).

Table 1. Mean of indicator organisms in treated and untreated wastewater released from Ayder referral hospital, Mekelle, North Ethiopia (September-December 2015).

Indicator organism	UHWW (n = 20)	THWW (n = 20)
Total heterotrophic count (CFU/mL)	1.9×10^8	1.6×10^6
Total coliform count (CFU/100mL)	2.6×10^{10}	2.2×10^6
Fecal coliform count (CFU/100mL)	1.25×10^9	2.0×10^5
E. coli count (CFU/100mL)	4.5×10^5	1.1×10^4

Table 2. Frequency of bacterial isolates from treated and untreated wastewater released from Ayder referral hospital, Mekelle, North Ethiopia (September-December 2015).

Bacteria isolates	UHWW (n = 20)	THWW (n = 20)	Total (n = 40)
S. aureus	13 (15.5%)	8 (16%)	21 (15.7%)
CoNS*	6 (7.1%)	5 (10%)	11 (8.2%)
Klebsiella spp	14 (16.7%)	10 (20%)	24 (17.9%)
P. aeruginosa	12 (14.3%)	8 (16%)	20 (14.9%)
E. coli	11 (13.1%)	6 (12%)	17 (12.7%)
Salmonella spp	9 (10.7)	6 (12%)	15 (11.2%)
Shigella spp	3 (3.6%)	0 (0)	3 (2.2%
Citrobacter spp	6 (7.1%)	3 (6%)	9 (6.7%)
Enterobacter spp	4 (4.8%)	2 (4%)	6 (4.5%)
Other isolates	6 (7.1%)	2 (4%)	8 (6%)
Total	**84 (100%)**	**50 (100%)**	**134 (100%)**

*CoNS: Coagulase negative *staphylococci,* other isolates: *Acinitobacter* spp (only 1 in UHWW), *Seratia* spp (3 in UHWW and 1 in THWW) and *Proteus* spp (2 in UHWW and 1 in THWW).

3.3. Drug Susceptibility Profile of Bacterial Isolates

Among bacterial isolates from untreated wastewater, Coagulase negative staphylococci (CoNS) were found to be 100% resistant to Penicillin and 50% to Cefoxitin. *S. aureus* was also found to be 77% and 38% resistant for Penicillin and Cefoxitin respectively. All isolates of *E. coli* and *Klebsiella* spp were 100% resistant to ampicillin (Table 3).

The total resistance of bacterial isolates from untreated wastewater was higher for penicillin 16/19 (84%) followed by ampicillin 42/52 (81%) and tetracycline 30/72 (42%). However, relatively lower resistance was observed among bacterial isolates to Chloramphenicol 13/71 (18%), gentamycin 19/84 (23%) and ciprofloxacin 20/84 (24%) (Figure 1).

Among bacterial isolates from treated wastewater, all isolates of *S. aureus* and CoNS were 100% resistant to penicillin. All isolates of *E. coli*, *Klebsiella* spp and *Citrobacter* spp were 100% resistant to ampicillin as shown in the (Table 4).

The total resistance of isolates from treated wastewater to penicillin was 13/13 (100%) followed by ampicillin 24/29 (83%) and Ceftriaxone 17/29 (59%). Relatively lower resistance among bacterial isolates was observed to ciprofloxacin 10/50 (20%), amikacin 10/50 (20%) and chloramphenicol 10/42 (24%) (Figure 2).

Among isolates from untreated wastewater, 19/72 (26.3%) were resistant for six and more antibiotics (e.g. three isolates of *klebsiella* spp and one isolates of *E. coli* were resistant to nine antibiotics; two isolates of *klebsiella* spp, two isolates of *E. coli* and one isolates of *Citrobacter* spp were resistant to eight antibiotics), while 13/84 (15.5%) isolates were resistant for only one antibiotic, and 7/84 (8.3%) were not resistant for any of antibiotics tested (Table 5).

Table 3. Drug Susceptibility profile of bacterial isolates from untreated wastewater released from Ayder referral hospital, Mekelle, North Ethiopia (September-December 2015).

Bacteria isolates		Antibiotics used N (%)												
		GN	AK	TTC	DO	SXT	CIP	CAF	AMP	CRO	AMC	E	P	CX
	R	2 (15)	4 (31)	3 (23)	1 (8)	2 (15)	2 (15)	4 (31)	NT	NT	NT	3 (23)	10 (77)	5 (38)
S. aureus (13)	**I**	3 (23)	3 (23)	4 (31)	6 (46)	3 (23)	2 (15)	0 (0)	NT	NT	NT	1 (8)	-	-
	S	8 (62)	6 (46)	6 (46)	6 (46)	8 (62)	9 (69)	9 (69)	NT	NT	NT	9 (69)	3 (23)	8 (62)
	R	0 (0)	1 (17)	3 (50)	2 (33)	2 (23)	1 (17)	1 (17)	NT	NT	NT	2 (33)	6 (100)	3 (50)
CoNS (6)	**I**	3 (50)	3 (50)	2 (23)	0 (0)	1 (17)	2 (23)	2 (23)	NT	NT	NT	2 (33)	-	-
	S	3 (50)	2 (33)	1 (17)	4 (66)	3 (50)	3 (50)	3 (50)	NT	NT	NT	2 (33)	0 (0)	3 (50)
	R	6 (43)	6 (43)	9 (64)	7 (50)	6 (43)	4 (29)	2 (14)	14 (100)	6 (43)	5 (36)	NT	NT	NT
Klebsiella spp (14)	**I**	2 (14)	3 (21)	1 (7)	3 (21)	2 (14)	3 (21)	4 (29)	0 (0)	3 (21)	2 (14)	NT	NT	NT
	S	6 (43)	5 (36)	4 (29)	4 (29)	6 (43)	7 (50)	8 (57)	0 (0)	5 (36)	7 (50)	NT	NT	NT
	R	2 (18)	2 (18)	4 (36)	2 (18)	5 (45)	3 (27)	1 (9)	11 (100)	4 (36)	3 (27)	NT	NT	NT
E. coli (11)	**I**	3 (27)	2 (18)	3 (27)	3 (27)	2 (18)	3 (27)	2 (18)	0 (0)	2 (18)	2 (18)	NT	NT	NT
	S	6 (55)	7 (64)	4 (36)	6 (55)	4 (36)	5 (45)	8 (73)	0 (0)	5 (45)	6 (55)	NT	NT	NT
	R	4 (33)	4 (33)	NT	NT	NT	3 (25)	NT	NT	NT	NT	NT	NT	NT
P. aeruginosa (12)	**I**	2 (17)	2 (17)	NT	NT	NT	4 (33)	NT	NT	NT	NT	NT	NT	NT
	S	6 (50)	6 (50)	NT	NT	NT	5 (42)	NT	NT	NT	NT	NT	NT	NT
	R	0 (0)	1 (11)	3 (33)	2 (22)	1 (11)	2 (22)	1 (11)	5 (56 (	3 (33)	3 (33)	NT	NT	NT
Salmonella spp (9)	**I**	4 (44)	2 (22)	2 (22)	2 (22)	3 (33)	2 (22)	3 (33)	2 (22)	2 (22)	0 (0)	NT	NT	NT
	S	5 (56)	6 (67)	4 (44)	5 (56)	5 (56)	5 (56)	5 (56)	2 (22)	4 (44)	6 (67)	NT	NT	NT
	R	1 (33	0 (0)	0 (0)	0 (0)	1 (33)	1 (33)	1 (33)	2 (67)	2 (67)	1 (33)	NT	NT	NT
Shigella spp (3)	**I**	0 (0)	1 (33)	1 (33)	1 (33)	1 (33)	0 (0)	0 (0)	0 (0)	0	0 (0)	NT	NT	NT
	S	2 (67)	2 (67)	2 (67)	2 (67)	1 (33)	2 (67)	2 (67)	1 (33)	1 (33)	2 (67)	NT	NT	NT
	R	2 (33)	2 (33)	3 (50)	2 (33)	2 (33)	2 (33)	1 (17)	5 (83)	3 (50)	1 (17)	NT	NT	NT
Citrobacter spp (6)	**I**	1 (17)	0 (0)	1 (17)	1 (17)	1 (17)	0 (0)	2 (33)	1 (17)	1 (17)	3 (50)	NT	NT	NT
	S	3 (50)	4 (67)	2 (33)	3 (50)	3 (50)	4 (67)	3 (50)	0 (0)	2 (33)	2 (33)	NT	NT	NT
	R	1 (25)	1 (25)	2 (50)	1 (25	1 (25)	1 (25)	1 (25)	2 (50)	0 (0)	1 (25)	NT	NT	NT
Enterobacter spp (4)	**I**	2 (50)	3 (75)	0 (0)	2 (50)	3 (75)	3 (75)	0 (0)	1 (25)	3 (75)	2 (50)	NT	NT	NT
	S	1 (25)	0 (0)	2 (50)	1 (25)	0 (0)	0 (0)	3 (75)	1 (25)	1 (25)	1 (25)	NT	NT	NT
	R	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	NT	NT	1 (100)	NT	NT	NT	NT
Acinetobacter spp (1)	**I**	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	NT	NT	0 (0)	NT	NT	NT	NT
	S	0 (0)	1 (100	0 (0)	0 (0)	0 (0)	0 (0)	NT	NT	0 (0)	NT	NT	NT	NT
	R	1 (20)	1 (20)	2 (40)	1 (20)	1 (20)	1 (20)	1 (20)	3 (60)	2 (40)	2 (40)	NT	NT	NT
Other isolates (5)	**I**	2 (40)	3 (60)	1 (20)	2 (40)	3 (60)	3 (60)	3 (60)	1 (20)	0 (0)	1 (20)	NT	NT	NT
	S	2 (40)	1 (20)	2 (20)	2 (40)	1 (20)	1 (20)	1 (20)	1 (20)	3 (60)	2 (40)	NT	NT	NT

GN: Gentamycin, **TTC**: Tetracyclin, **SXT**: Cotrimoxazole, **CAF**: Chloramphenicol, **P**: Penicillin, **AK**: Amikacin, **DO**: Doxycycline, **CIP**: Ciprofloxacin, **E**: Erytromyacin, **CX**: Cefoxitin, **AMC**: Amoxacillin-clavulunic acid, **AMP**: Ampicillin, **CRO**: Ceftriaxone, **R**: Resistance, **S**: Susceptible, **I**, Intermediate, **NT**: Not Tested, **Other isolates**: *Seratia* spp (3) and *Proteus* spp (2).

Table 4. Drug Susceptibility profile of bacterial isolates from treated wastewater released from Ayder referral hospital, Mekelle, North Ethiopia (September-December 2015).

Bacteria isolates		Antibiotics used N (%)												
		GN	AK	TTC	DO	SXT	CIP	CAF	AMP	CRO	AMC	E	P	CX
	R	2 (25)	1 (13)	2 (25)	3 (38)	1 (13)	1 (13)	2 (25)	NT	NT	NT	3 (38)	8 (100)	3 (38)
S. aureus (8)	**I**	1 (13)	4 (50)	3 (38)	2 (25)	4 (50)	3 (38)	1 (13)	NT	NT	NT	1 (13)	-	-
	S	5 (63)	3 (38)	3 (38)	3 (38)	3 (38)	4 (50)	5 (63)	NT	NT	NT	4 (50)	0 (0)	5 (63)
	R	1 (20)	0 (0)	2 (40)	2 (40)	1 (20)	0 (0)	2 (40)	NT	NT	NT	1 (20)	5 (100)	2 (40)
CoNS (5)	**I**	3 (60)	2 (40)	0 (0)	0 (0)	0 (0)	2 (40)	0 (0)	NT	NT	NT	3 (60)	-	-
	S	1 (20)	3 (60)	3 (60)	3 (60)	4 (80)	3 (60)	3 (60)	NT	NT	NT	1 (20)	0 (0)	3 (60)
	R	6 (60)	4 (40)	4 (40)	4 (40)	6 (60)	2 (20)	2 (20)	10 (100)	5 (50)	3 (30)	NT	NT	NT
Klebsiella spp (10)	**I**	0 (0)	1 (10)	2 (20)	3 (30)	0 (0)	4 (40)	3 (30)	0 (0)	1 (10)	3 (30)	NT	NT	NT
	S	4 (40)	5 (50)	4 (40)	3 (30)	4 (40)	4 (40)	5 (50)	0 (0)	4 (40)	4 (40)	NT	NT	NT
	R	2 (33)	2 (33)	2 (33)	2 (33)	4 (67)	2 (33)	1 (17)	6 (100)	3 (50)	3 (50	NT	NT	NT
E. coli (6)	**I**	1 (17)	0 (0)	0 (0)	1 (17)	0 (0)	1 (17)	1 (17)	0 (0)	2 (33)	1 (17)	NT	NT	NT
	S	3 (50)	4 (67)	4 (67)	3 (50)	2 (33)	3 (50)	4 (67)	0 (0)	1 (17)	2 (33)	NT	NT	NT
	R	4 (50)	2 (25)	NT	NT	NT	3 (38)	NT	NT	NT	NT	NT	NT	NT
P. aeruginosa (8)	**I**	2 (25)	4 (50)	NT	NT	NT	0 (0)	NT	NT	NT	NT	NT	NT	NT
	S	2 (25)	2 (25)	NT	NT	NT	5 (62)	NT	NT	NT	NT	NT	NT	NT
	R	2 (33)	0 (0)	3 (50)	2 (33)	2 (33)	1 (17)	1 (17)	3 (50)	4 (67)	2 (33)	NT	NT	NT
Salmonella spp (6)	**I**	1 (17)	2 (33)	0 (0)	0 (0)	1 (17)	2 (33)	2 (33)	0 (0)	0 (0)	1 (17)	NT	NT	NT
	S	3 (50)	4 (67)	3 (50)	4 (67)	3 (50)	3 (50)	3 (50)	3 (50)	2 (33)	3 (50)	NT	NT	NT
	R	1 (33)	1 (33)	3 (100)	2 (67)	1 (33)	1 (33)	1 (33)	3 (100)	2 (67)	2 (67)	NT	NT	NT
Citrobacter spp (3)	**I**	2 (67)	0 (0)	0 (0)	0 (0)	2 (67)	0 (0)	0	0 (0)	1 (33)	0 (0)	NT	NT	NT
	S	0 (0)	2 (67)	0 (0)	1 (33)	0 (0)	2 (67)	2 (67)	0 (0)	0 (0)	1 (33)	NT	NT	NT
	R	0 (0)	0 (0)	1 (50)	1 (50)	1 (50)	0 (0)	0	1 (50)	1 (50)	1 (50	NT	NT	NT
Enterobacter spp (2)	**I**	2 (100)	2 (100)	1 (50)	0 (0)	0 (0)	2 (100)	1 (50)	1 (50)	0 (0)	1 (50)	NT	NT	NT
	S	0 (0)	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)	NT	NT	NT
	R	1 (50)	0 (0)	2 (100)	1 (50)	1 (50)	0 (0)	1 (50)	1 (50)	2 (100)	2 (100)	NT	NT	NT
Other isolates (2)	**I**	1 (50)	1 (50)	0 (0)	0 (0)	1 (50)	2 (100)	0	1 (50)	0 (0)	0 (0)	NT	NT	NT
	S	0 (0)	1 (50)	0 (0)	1 (50)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	NT	NT	NT

GN: Gentamycin, **TTC**: Tetracyclin, **SXT**: Cotrimoxazole, **CAF**: Chloramphenicol, **P**: Penicillin, **AK**: Amikacin, **DO**: Doxycycline, **CIP**: Ciprofloxacin, **E**: Erytromyacin, **CX**: Cefoxitin, **AMC**: Amoxacillin-clavulunic acid, **AMP**: Ampicillin, **CRO**: Ceftriaxone **R**: Resistance, **S**: Susceptible, **I**, Intermediate, **NT**: Not Tested, Other isolates: *Seratia* spp (1) and *Proteus* spp (1).

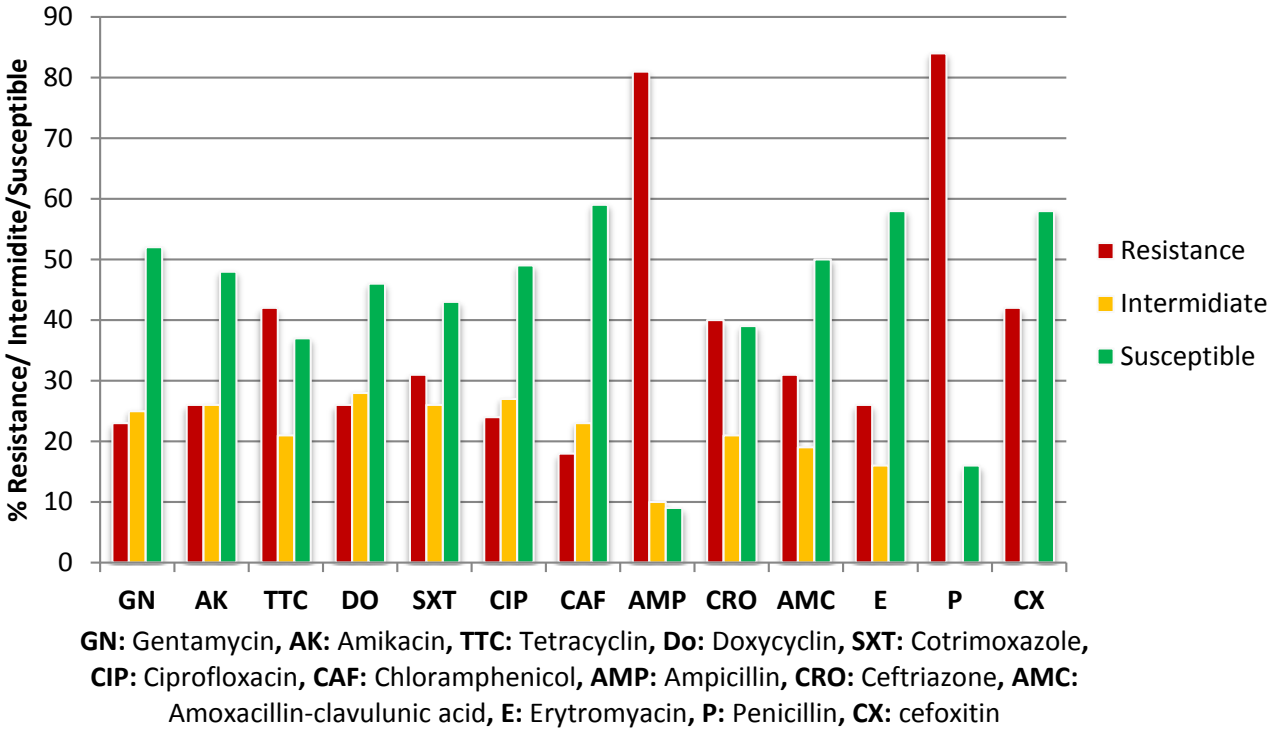

Figure 1. Total drug susceptibility profile of isolates from untreated wastewater released from Ayder referral hospital, Mekelle, North Ethiopia (September-December 2015).

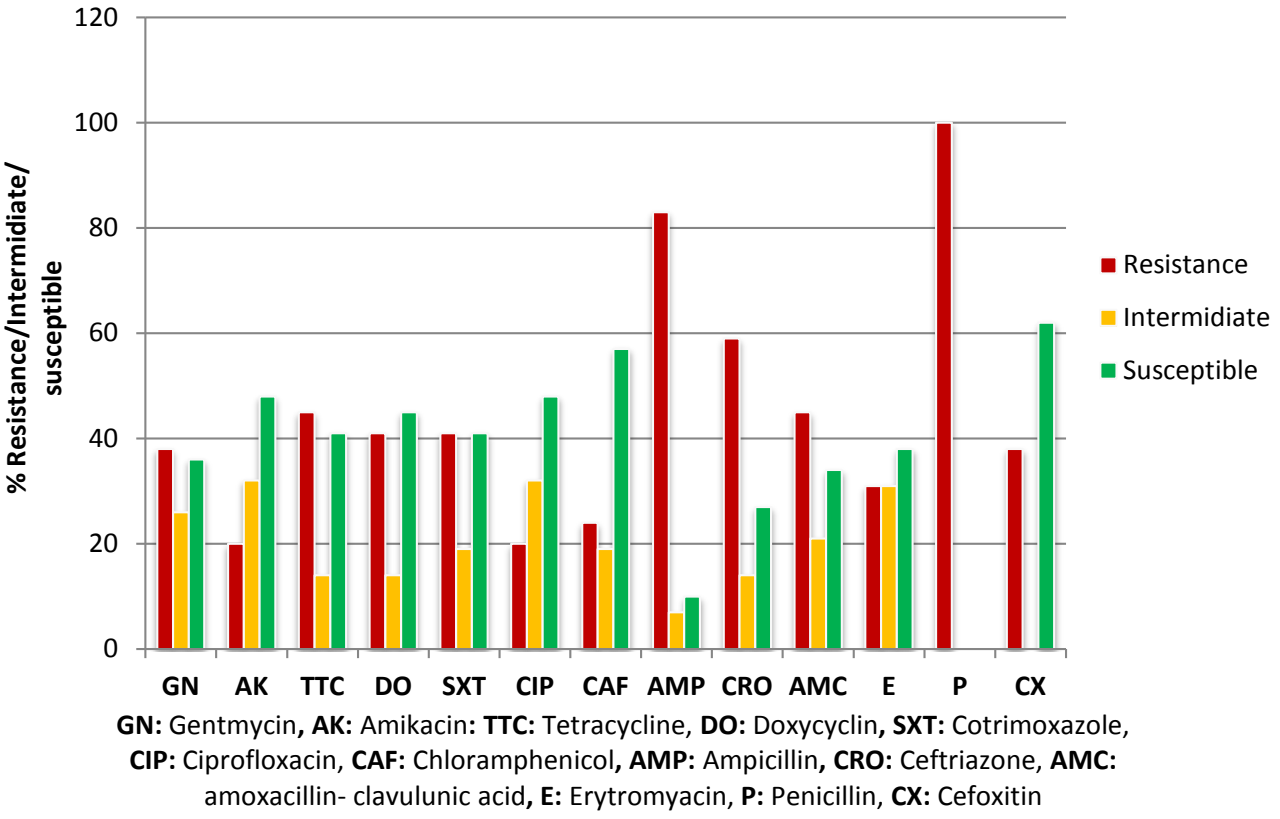

Figure 2. Total drug susceptibility profile of isolates from treated wastewater released from Ayder referral hospital, Mekelle, North Ethiopia (September-December 2015).

Table 5. Multi-drug resistance patterns of bacterial isolates from untreated wastewater released from Ayder Referral Hospital, Mekelle, North Ethiopia (September-December 2015).

Bacteria isolates	Number of isolates	R0	R1	R2	R3	R4	R5	≥R6
S. aureus	13	0 (0)	3 (23.1)	4 (30.8)	3 (23.1)	0 (0)	1 (7.7)	2 (15.4)
CoNS	6	0 (0)	0 (0)	3 (50)	1 (16.7)	1 (16.7)	0 (0)	1 (16.7)
Klebsiella spp	14	0 (0)	1 (7.1)	3 (21.4)	1 (7.1)	0 (0)	1 (7.1)	8 (57.1)
E. coli	11	0 (0)	2 (18.2)	2 (18.2)	1 (9.1)	1 (9.1)	2 (18.2)	3 (27.3)
P. aeroginosa	12	5 (41.7)	2 (18.2)	3 (25)	2 (18.2)	NT	NT	NT
Salmonella spp	9	1 (11.1)	0 (0)	2 (22.2)	1 (11.1)	1 (11.1)	2 (22.2)	2 (22.2)
Shigella spp	3	1 (33.3)	1 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (33.3)
Citrobacter spp	6	0 (0)	2 (33.3)	0 (0)	0 (0)	0 (0)	2 (33.3)	2 (33.3)
Entrobacter spp	4	0 (0)	2 (50.0)	1 (25)	0 (0)	1 (25)	0 (0)	0 (0)
Other isolates	6	0 (0)	0 (0)	2 (33.3)	1 (16.7)	2 (66.7)	1 (016.7)	0 (0)
Total	84	**7 (8.3)**	**13 (15.5)**	**20 (23.8)**	**10 (11.9)**	**6 (8.3)**	**9 (12.5)**	**19 (26.4)**

R0: Not resistant for any antibiotics tested, R1: Resistant to one antibiotic, R2: Resistant to two antibiotics, R3: Resistant to three antibiotics, R4: Resistant to four antibiotics, R5: Resistant to five antibiotics, ≥R6: Resistant to six or more antibiotics, NT: Not Tested, Other isolates: *Accinitobacter* spp (1), *Seratia* spp (3) and *Proteus* spp (2).

Among isolates from treated wastewater, 12/42 (28.6%) were resistant for six and more antibiotics (e.g. two isolates of *Klebsiella* spp and one isolates of CoNS were resistant for ten antibiotics, one isolates of *Citrobacter* spp were also resistant for nine antibiotics), while 8/50 (16%) were resistant for only one antibiotics and there was no isolates that are not resistant for any of antibiotics tested (Table 6).

Among isolates from both treated and untreated hospital wastewater the overall prevalence of multi-drug resistance (resistant to two and above antibiotics) in this study was found to be 106/134 (79.1%). Multi-drug resistance in untreated wastewater sample was found to be 64/84 (76.2%) whereas in treated wastewater was found to be 42/50 (84%). In this study isolates from treated wastewater was found to be 1.64 times resistant for many drug than isolates from untreated hospital wastewater (COR:1.64, 95% CI: 1.15 - 3.29, P: 0.001).

4. Discussion

The present study showed that mean heterotrophic plate count (1.6×10^6 CFU/mL), was exceeded the permissible limit of Environment Protection Agency, EPA [16] and Health Protection Agency, HPA [17] (<1000 CFU/mL) for treated wastewater. Total coliform count (2.2×10^6 CFU/100 mL) also failed to fulfill the requirements of the revised guidelines on the quality of treated wastewater used in agriculture, in public parks ($<5 \times 10^3$ CFU/100mL) [18] [19].

Table 6. Multi-drug resistance patterns of bacterial isolates from treated wastewater released from Ayder Referral Hospital, Mekelle, North Ethiopia (September-December 2015).

Bacteria isolates	Number of isolates	R0	R1	R2	R3	R4	R5	≥R6
S. aureus	8	0 (0)	1 (12.5)	2 (25)	2 (25)	1 (12.5)	0 (0)	2 (25)
CoNS	5	0 (0)	0 (0)	0 (0)	1 (20)	2 (40)	1 (20)	1 (20)
Klebsiella spp	10	0 (0)	1 (10)	2 (20)	2 (20)	1 (10)	1 (10)	3 (30)
E. coli	6	0 (0)	1 (16.7)	0 (0)	1 (16.7)	2 (33.3)	0 (0)	2 (33.3)
P. aeroginosa	8	0 (0)	5 (62.5)	1 (12.5)	2 (25)	NT	NT	NT
Salmonella spp	6	0 (0)	0 (0)	1 (16.7)	2 (33.3)	1 (16.7)	0 (0)	2 (33.3)
Citrobacter spp	3	0 (0)	0 (0)	1 (33.3)	0 (0)	0 (0)	0 (0)	2 (66.7)
Entrobacter spp	2	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	1 (50)	0 (0)
Other isolates	2	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)
Total	50	**0 (0)**	**8 (16)**	**8 (16)**	**10 (20)**	**8 (19.0)**	**4 (9.5)**	**12 (28.6)**

R0: Not resistant for any antibiotics tested, R1: Resistant to one antibiotic, R2: Resistant to two antibiotics, R3: Resistant to three antibiotics, R4: Resistant to four antibiotics, R5: Resistant to five antibiotics, ≥R6: Resistant to six or more antibiotics, NT: Not Tested, Other isolates: *Seratia* spp (1) and *Proteus* spp (1).

Microbial contamination of treated hospital wastewater was 2.0×10^5 of fecal coliforms, which was higher than WHO maximum tolerable limit for fecal indicator bacteria ($\leq 10^3$/100 mL for unrestricted irrigation and $\leq 10^5$/100 mL for restricted irrigation) [20] [21]. Faecal coliform count was also higher than FAO standard allowable limit of 5000 CFU/100 mL [22]. High density of *E. coli* (1.1×10^4) in treated wastewater was also an indication of fecal pollution of environment due to human activities.

In the present study bacterial isolates such as *Klebsiella* spp 10 (20%), *P. aeruginosa* 8 (16%), *S. aureus* 8 (16%), *E. coli* 7 (14%) and *salmonella* spp 6 (12%) were also frequently detected in treated wastewater. The same study conducted in Ethiopia, Hawassa University Referral Hospital, reported *Salmonella* spp, *Shigella* spp, *E. coli* and *S. aureus* from hospital effluent [3]. Similarly, from Thailand pathogenic bacteria like *Vibrio* spp and *Salmonella* spp as well as other potentially pathogenic bacteria were reported [23]. Likewise, study in India showed large numbers of enteric-bacteria, *S. aureus* and *P. aeruginosa* [24]. Study in Tunisia also showed large amount of pathogenic bacteria like *Salmonella* that have a real impact in human health [25]. The highest prevalence of Salmonella species is due to the fact that it is highly associated with typhoid fever, paratyphoid fever and gastroenteritis, especially of developing countries. Different research also revealed that its survival in fresh water and sewage is less than 60 days and better recovered from sewage system compared to *Shigella*. The highest microbial contamination of treated wastewater shows wastewater treatment plant may not be efficient with regard to removing bacteria in the final effluent that is discharged to the receiving environment.

Among bacterial isolates from treated wastewater, *S. aureus* and CoNS were 100% resistant to penicillin and also *E. coli*, *Klebsiella* spp and *Citrobacter* spp were 100% resistant to ampicillin. In general most bacterial isolates were highly resistant to tetracycline, doxycycline, cotrimoxazole, amoxicillin-clavulinic acid and ceftriaxone. The same study in India showed simultaneous resistance of isolates for ampicillin, ampicillin with clavulinic acid, cotrimoxazole, tetracycline, first, second and third generation cephalosporins in the final effluent of wastewater treatment plant [26]. Study in Alexandria, Egypt also showed the presence of antibiotic resistant extended spectrum B-lactamase (ESBL) producing bacteria at the end of wastewater purification process [2], posing a risk of its spread to the environment and subsequent human and animal exposure.

Overall resistance of bacterial isolates from THWW for methicillin was found to be 38%. Methicillin resistant *Staphylococci* (MRSA and MRCoNS) were not studied well in our country from hospital effluents. But as indicated by Abulreesh [27] from International Conference on biotechnology and environment management, Singapore multidrug resistant staphylococci (*S. aureus* and coagulase negative *Staphylococci*) had been a common problem and recovered from diverse environmental sources, such as drinking water supplies and hospital environments which warns public health concern since contamination of river and lake with this pathogen may pose risk to the public health associated with *Staphylococcal* infection and food poisoning.

This study also observed that, most bacterial isolates from the THWW shows higher rate of resistance than bacterial isolates from the UHWW and shows resistant bacteria isolates were able to survive the journey to the inlet of sewage treatment plant and treatment process. The same result was reported from Alice, Eastern Cape province of South Africa [28] and European countries [29] [30], where higher rate of resistance in bacterial isolates from final effluent of wastewater treatment plant was found. Presences of high percentage of drug resistant isolates from the final effluent of WWTP suggest that, hospital wastewater could have contributed massively to the resistances observed among the isolates from the final effluent. These can be due to the fact that, hospital wastewater contains a diverse group of pathogenic, commensal and environmental bacteria. This characteristic composition makes sewage particularly suitable ecological niche for the growth and spread of antibiotic resistance due to selection pressure and horizontal gene transfer [31] [32] [33].

High percentages of multi-drug resistance for the majority of the isolates in THWW were discharged to the environment. This was supported by Study conducted in Switzerland which showed that increased proportions of highly and extremely multi-resistant bacteria among the isolated sulfamethoxazole/trimethoprim and streptomycin resistant strains in the sample of treated wastewater compared to the wastewater treatment plant inlet sample [34]. Study conducted in Australia [35], Brazil [36] and China [37] that investigates a hospital sewage treatment system also showed, the treatment of the hospital wastewater may not be totally effective in removing multiple drug resistant bacteria and

resistant gene from hospital wastewater.

The release of MDR bacteria to the environment causes extensive genetic exchange; where opportunistic pathogens (commonly found in free-living communities) may become resistant upon acquiring resistance mechanisms. Therefore, reduction of selective pressure by regulating the use of antibiotics is a key step to undermine the spread of resistance in hospital wastewater in order not to favored resistant strains [38].

5. Conclusion

In the present study, high numbers of indicator organisms were obtained from treated hospital wastewater, which exceeded the WHO, HPA, EPA and FAO standard permissible levels. Significant pathogenic and potentially pathogenic bacteria were also isolated from the treated wastewater. There was high prevalence of drug resistant isolates from untreated and treated hospital wastewater suggesting their persistence in the hospital environment, and their ability to pass the processes of treatment plant. Therefore Liquid waste treatment system (Chlorination) should be developed to disinfect pathogens in treated wastewater effluents.

Ethical Approval

Approved.

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

TA participated in its design and performed the laboratory activities. **TA** analyzed the data and wrote the manuscript. **LN**, **AK** and **YW** reviewed the manuscript. All authors read and approved the final manuscript.

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