



EXTENDED-SPECTRUM BETA-LACTAMASE-PRODUCING *Enterobacteriaceae* IN EFFLUENTS OF DIFFERENT HOSPITALS SEWAGE IN BIRATNAGAR, NEPAL

Sanjay Mahato^{1,2}, Ajay Mahato³ and Prajjwal Adhikari²

¹AASRA Research and Education Academy Counsel, Biratnagar - 6, Nepal

²Department of Microbiology, Mahendra Morang Adarsha Multiple Campus, Tribhuvan University, Nepal

³Department of Orthopaedics, Birat Medical College and Teaching Hospital, Biratnagar, Nepal

*Corresponding author: mahato.sanjay@gmail.com

Abstract

Untreated water from hospitals of Biratnagar as effluent is a serious concern from health point of view. Antimicrobial resistant bacteria are a serious threat which may contaminate the drinking water and environment. With an aim of isolation and detection of Multidrug resistance (MDR) and Extended-spectrum β - lactamases (ESBL) producing *Enterobacteriaceae* from the effluents and sewage samples of hospitals, this study builds the importance to inquiry about the involvement of hospital liquid waste discharge in the development and distribution of antibiotics resistance in the environment bacteria. Ten hospital sewage samples were aseptically collected, processed and analyzed. Isolates were biochemically identified, and their antimicrobial activity were tested. Of the ten sewage samples analyzed, 23 bacteria isolates were isolated which contained 8 *Escherichia coli* (34.7%), 5 isolates each of *Citrobacter* spp (21.7%) and *Enterobacter* spp (21.7%), 3 isolates of *Klebsiella* spp (13%), and 1 isolate each of *Shigella* spp (4.3%) and *Yersinia* spp (4.3%). Most of the bacteria isolated were resistant to ampicillin, ceftazidime, cefpodoxime, amoxicillin/clavulanate, cefotaxime, cefoxitin, ceftriaxone, and cefuroxime. The isolates were sensitive to nitrofurantoin, azithromycin, trimethoprim/sulfamethoxazole, chloramphenicol, ciprofloxacin, ofloxacin, and gentamycin antibiotics. Out of twenty-three isolates, 16 (69.6%) were found to be multidrug resistant, 7 (30.4%) were producing extended beta lactamase, while 18 (78.3%) multi-antibiotics resistance index greater than 20%. Among the bacteria isolated; 80% of the *Citrobacter*; and 75% of the *E. coli* were found biofilm producing bacteria. Sewage treatment plant must be established in hospital for their effluents and sludge coming from the hospital.

Keywords: Biofilm; Drug resistance; *Enterobacteriaceae*

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Introduction

Hospital wastewater can be hazardous to public health as a result of pollutants, pharmaceutical wastes and pathogenic microorganisms that are antibiotics resistant (Moges et al., 2014). Antibiotics exposure provides selective pressure to bacteria, which makes the surviving bacteria more likely to be resistant. Bacteria that were at one time susceptible to an antibiotic can acquire resistance through mutation of their genetic material or by acquiring fragments of DNA that code for the resistance properties from other bacteria. Sewage is the byproduct of water characterized by physical, chemical, and bacteriological contaminants. Uncontrolled and excessive use of antibiotics by human cause increase in multidrug resistance in hospital effluents (Hauhnar et al., 2018). One milliliter of sewage typically contains between 10^5 and 10^6 microorganisms (Shchegolkova et al., 2016). *Enterobacteriaceae* is a large family of gram-negative bacteria that includes many of the more familiar pathogens such as *E. coli*, *Enterobacter*, *Salmonella*, *Shigella*, *Serratia*, *Yersinia*, *Klebsiella*, *Citrobacter*, and *Proteus*. These bacteria are found in the environment and are also normal biota of human and animal intestines (Munita and Arias, 2016). They are rod-shaped bacilli, non-spore forming, facultative anaerobes, and are motile with peritrichous flagella while some species are non-motile (Exner et al., 2017). β -lactam antibiotics contain a beta-lactam ring in their molecular structure. This includes penicillin derivatives, cephalosporins, monobactams, carbapenems and carbacephems (Pandey and Cascella, 2019). Multidrug resistance (MDR) is defined as an isolate that is not susceptible to at least one agent in at least three antimicrobial classes. Extended-spectrum β -lactamases (ESBL) are enzymes that destroy the active sites of extended-spectrum third generation cephalosporins and monobactams thereby making bacteria resistant to such important antibiotics (Mahato et al., 2018). ESBLs are derived from genes TEM, SHV, and CTX-M by mutations that alter the amino acid configuration around the enzyme active site (Bajpai et al., 2017). ESBLs producing *Enterobacteriaceae* are prevalent worldwide especially in clinical environment. Biofilm is the structure formed by adhesion of bacterial cells to cells and cells to surface. Adherent cells become embedded within slimy extracellular matrix. Extracellular polymeric substances (EPS) is composed of extracellular polysaccharides, proteins, lipids and DNA. Biofilms may be formed on living or non-living substances by the same species or may be of different species (Martino, 2018). Biofilm provides defense from physical forces, phagocytosis and penetration of antimicrobial, provides microniche so that they can communicate, helps them in gene transfer as well as increased food availability (Stewart et al., 2015).

The main aim of this study was to determine the prevalence of MDR and ESBL producing *Enterobacteriaceae* from the various samples collected from the effluents and sewage of hospital. This study would investigate about the need of sterilization of hospital wastewater before discharge; investigate the

involvement of hospital effluents in the distribution of antibiotics resistant bacteria like *Enterobacteriaceae* which cause serious public health problem; and elucidate baseline information that could be utilized for formulating guidelines for the treatment of hospital sewages. It was hypothesized that there is no association between the type of bacterial strains of *Enterobacteriaceae* and their response to the antibiotics.

Materials and methods

Sample collection and handling

A total of 10 sewage samples were aseptically collected from hospitals (Table 1) in a sterile high-density polyethylene sample bottle (HDPE) of a capacity of 500 ml during the period of March to October 2018 (Chattopadhyay, 2017). To ensure that well mixed samples were collected, samples were collected near the center of flow channel, at approximately 10-15 centimeter of the water depth, where the turbulence was at maximum and the possibility settling was minimized. Skimming the water surface or dragging the bottle was evaded. The samples were taken with the help of test tube. The sewage water was mixed before sampling. After taking the sample, the neck of the bottle was wiped with 95% alcohol. Each sample bottle was labelled with date, code number, and time. Placing the collected sewage samples on 4° C ice box, samples were transported (within 2 hours) to Microbiology laboratory of Mahendra Morang Adarsh Multiple Campus, Biratnagar and were analyzed on the same day on culture media plates. Samples were refrigerated if the analysis was delayed. Distilled water was used as control during analysis.

Table 1: Name of hospital and Wastewater sample location with microbes isolated

S. N.	Name of Hospital	Sampling Location	Organisms isolated
1	Tulasa Mother and Child Hospital	Outside gate	1. <i>Enterobacter</i> 2. <i>Klebsiella</i> 3. <i>E. coli</i>
2	R. K. Hospital	South side	1. <i>Enterobacter</i> 2. <i>E. coli</i>
3	R. K. Hospital	West side	1. <i>E. coli</i> 2. <i>Citrobacter</i>
4	Koshi Zonal Hospital	Outside	1. <i>E. coli</i> 2. <i>Yersinia</i> 3. <i>Citrobacter</i> 4. <i>Klebsiella</i>
5	Koshi Zonal Hospital	Inside	1. <i>E. coli</i>
6	Trauma Centre	Outside gate	1. <i>Enterobacter</i> 2. <i>Citrobacter</i>

7	Saptakoshi Hospital	Inside gate	1. <i>Citrobacter</i> 2. <i>E. coli</i>
8	Saptakoshi Hospital	Outside gate	1. <i>Citrobacter</i> 2. <i>E. coli</i>
9	Birat Eye Hospital	Gate	1. <i>Enterobacter</i> 2. <i>Klebsiella</i>
10	Biratnagar Eye Hospital	Gate	1. <i>Enterobacter</i> 2. <i>E. coli</i> 3. <i>Shigella</i>

Isolation and Identification of Enterobacteriaceae

The wastewater samples collected from the hospital sewage were serially diluted (1 mL sample and 9 mL diluent) in 0.85% saline water (Health Protection Agency, 2013). A volume of 100 µl from each sample and 100 µL of the following serial dilutions (10^{-1} , 10^{-2} and 10^{-3}) in saline were inoculated onto Eosin Methylene Blue (EMB) Agar, MacConkey Agar, and Xylose Lysine Deoxycholate (XLD) Agar for *Enterobacteriaceae* family such as *E. coli*, *K. pneumoniae*, *Enterobacter* spp, *Citrobacter* spp, *Salmonella* spp, *Shigella* spp, and *Yersinia* spp. The inoculation on culture media was done by spread plate method and was incubated aerobically at 37 °C for 24 - 48 hours. After incubation, colonies were picked on their colony morphology like colonial appearance, size, elevation, color, margin, and opacity. The green metallic sheen colony on EMB agar indicated it to be *E. coli* while the pink colored mucoid and smooth colonies suggested *K. pneumoniae* strains. *K. pneumoniae* is pink in MacConkey agar whereas *K. oxytoca* is purple in color. Red colonies without black center were supposed to be *Shigella* spp. All the selected colonies were, then, sub-cultured on nutrient agar plate to obtain pure culture. The isolated organisms were identified microscopically and biochemically. Gram staining and capsule staining from the colonies was done for a preliminary identification of the pathogenic bacteria. Triple sugar iron (TSI), Sulfate/indole/motility (SIM), Methyl Red (MR) test, Voges – Proskauer (VP) test, and citrate agar, catalase test, oxidase test, and urea hydrolysis tests were performed to identify the organisms as per the guidelines in the Manual of Clinical Microbiology (Fig. 1) (Murray et al., 2007).

Antimicrobial susceptibility tests

Biochemically identified isolates of *Enterobacteriaceae* were subjected to antimicrobial susceptibility testing using Kirby-Bauer antibiotic testing according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2016). The suspensions turbidity of 0.5 McFarland standard was used for antimicrobial

sensitivity testing and inoculated onto Mueller- Hinton agar (MHA) medium. The 0.5 McFarland Standard is comparable to a bacterial suspension of 1.5×10^8 CFU/ml. The following antimicrobial disk (Himedia, India) were used: Ampicillin (AMP) (10 µg), Amoxicillin/Clavulanate (AMC) (20/10 µg), Cefoxitin (CX) (30 µg), Cefuroxime (CXM) (30 µg), Cefotaxime (CTX), Ceftazidime (CAZ) (30 µg), Ceftriaxone (CTR) (30 µg), Cefpodoxime (CPD) (10 µg), Gentamicin (GEN) (30 µg), Azithromycin (AZM) (15 µg), Ciprofloxacin (CIP) (5 µg), Ofloxacin (OF) (5 µg), Trimethoprim/sulfamethoxazole (COT) (1.25/23.75 µg), Chloramphenicol (C) (30 µg) and Nitrofurantoin (NIT) (300 µg). The swabbed MHA plates were impregnated with the discs and incubated at 37 °C for 24 hours. Zone of inhibition was measured in millimeter using scale. Using the standard chart (CLSI, 2016), the zone of inhibition measured was compared and then the organisms were reported as susceptible, intermediate, or resistant accordingly.

Criterion for multidrug resistance

Isolates which established the resistance to at least one agent in three or more classes of the drug *in-vitro* were defined as multidrug resistant (MDR) bacteria (CLSI, 2016; Magiorakos et al., 2011).

ESBL detection

Isolates exhibiting a zone of inhibition of growth for ceftazidime and ceftriaxone ≤ 22 mm and ≤ 25 mm, respectively, were presented to the combined disc test in order to check for ESBL- producing strains (Abayneh et al., 2018). The combined disc methodology used to detect ESBL- producing *Enterobacteriaceae* was performed in as described by CLSI (2016). The antibiotic discs (Himedia, India) used were ceftazidime (30 µg) and ceftazidime (30 µg) plus clavulanic acid (10 µg), and cefotaxime (30 µg) and cefotaxime (30 µg) plus clavulanic acid (10 µg). Results were interpreted according to the criteria established by the CLSI (2016). An increase of 5 mm in the zone of inhibition of growth for ceftazidime plus clavulanic acid as compared with the zone formed by the ceftazidime disc, and a 5 mm increase in the zone diameter for cefotaxime plus clavulanic acid as compared with the zone around the cefotaxime disc, were confirmatory for the result of ESBL- producing strains.

Multiple antibiotic resistance (MAR) index

MAR index is the number of antibiotics to which test isolate displayed resistance divided by the total number of antibiotics to which the test organism has been evaluated for sensitivity. MAR index for each isolate was calculated as per the guidelines of Al Momani et al. (2019). Isolates with a MARI of greater than 0.2 was accounted as Multi-Antibiotics Resistance strains while those with 0.2 to 0.25 was not considered because of chances of ambiguity.

Biofilm detection

Congo red agar (CRA) medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar No. 1 10 g/L and Congo Red indicator 0.8 g/L. First Congo Red stain was prepared as a concentrated aqueous solution and autoclaved (121 ° C for 15 minutes) separately from the other medium constituents (Triveda and Gomathi, 2016). Then it was added to the autoclaved brain heart infusion agar with sucrose at 55 ° C. CRA plates were inoculated with test organisms and incubated at 37 ° C for 24 hours aerobically. Black colony with a dry crystalline consistency indicated biofilm production. Single colony taken from an overnight grown culture was streaked on CRA and was incubated for 24 hours. After incubation, observation was noted. Dark colored area represented biofilm formation and non-dark area represented non-biofilm forming bacteria (Hassan et al., 2011).

Data Analysis

The data were statistically analyzed using Statistical Package for Social Sciences (SPSS v21) software package. Chi-square test at p-value <0.05 was considered statistically significant. The effect size's using Cramer's V test was calculated.

Results

A total of 10 samples were analyzed for the presence of *Enterobacteriaceae* family and 23 different types of bacteria were isolated. Out of 23 isolates, 8 (80%) samples contained *E. coli*, 5 (50%) of samples contained *Citrobacter* and *Enterobacter* each, 3 (30%) showed *Klebsiella*, 1 (10%) sample contained *Shigella* and *Yersinia*. All samples were tested positive and contained more than one type of isolates except one sample. The bacterial distribution in 23 isolates were *E. coli* (34.7%), *Citrobacter* (21.7%), *Enterobacter* (21.7%), *Klebsiella* (13%), *Shigella* (4.3%) and *Yersinia* (4.3%).

Green metallic sheen colonies on EMB agar were non-capsulated gram-negative bacilli named *E. coli*. Pink colored, highly mucoid colonies in EMB agar on further examinations were found to be gram negative capsulated bacilli *Klebsiella* spp (Table 2 and 3). Several mucoid pink colored colonies in MacConkey agar were found to be gram negative non-capsulated bacilli *Enterobacter* spp. Few mucoid or rough colonies in MacConkey Agar on further examinations were found to be *Citrobacter* spp with gram negative non-capsulated bacilli characteristics. From XLD agar, one pink colony without black center was found to be *Shigella* spp. The same culture showed pale and yellowish colonies on MacConkey agar. One strain was identified as capsulated *Yersinia* spp (Table 2 and 3).



Fig 1. Biochemical identification of *E. coli*



Fig 2. Antibiotics sensitivity test (AST) of *Escherichia coli*



Fig 3. ESBL detection method of *E. coli*

Table 2: Biochemical characteristics of bacteria

Citrate	Urease	Indole	MR	VP	Catalase	Oxidase	Motility	TSI	Gas/ H ₂ S	Results
+	-	-	-	+	+	-	+	A/A	+/-	<i>Enterobacter</i> spp
+	+	-	-	+	+	-	-	A/A	+/-	<i>Klebsiella</i> spp
-	-	+	+	-	+	-	+	A/A	+/-	<i>E. coli</i>
+	-	-	+	-	+	-	+	A/A or K/A	+/+	<i>Citrobacter</i> spp
-	+	+			+	-	+		-	<i>Yersinia</i> spp
-	-	-	+	-	+	-	-	K/A	-/-	<i>Shigella</i> spp

+ represents Positive test, - represents Negative test, while D denotes Variable result (either positive or negative), A and K signify Acidic and Alkaline respectively.

Table 3: Identified isolates with sample code

Sample Code	Isolates Identified
EBN S1, EPN S2, EPN S3, EGM S4, EGM S5, EGM S7, EGM S8, EGM S10	<i>E. coli</i>
EP S1, EPN S4, EM S9	<i>Klebsiella</i> spp
EPN S1, EP S2, EP S6, EP S9, EM S10	<i>Enterobacter</i> spp
EP S3, EP S4, EB S6, EP S7, EB S8	<i>Citrobacter</i> spp
EB S4	<i>Yersinia</i> spp
EP S10	<i>Shigella</i> spp

Table 4: Antibiotic resistance percentage of isolated bacteria

Antibiotics	Antibiotic Resistance (%)					
	<i>E. coli</i>	<i>Klebsiella</i> spp	<i>Enterobacter</i> spp	<i>Citrobacter</i> spp	<i>Yersinia</i> spp	<i>Shigella</i> spp
Ampicillin	100	100	100	100	100	100
Amoxicillin/ Clavulanate	75	100	100	60	100	100
Cefoxitin	62.5	33.3	100	100	0	100
Cefuroxime	62.5	33.3	80	80	0	100
Cefotaxime	75	0	100	80	0	100
Ceftazidime	87.5	100	100	80	100	100
Ceftriaxone	62.5	33.3	80	80	0	100
Cefpodoxime	87.5	33.3	100	80	100	100
Gentamicin	12.5	0	20	0	0	0
Azithromycin	25	0	40	20	0	0
Ciprofloxacin	12.5	33.3	20	20	0	0
Ofloxacin	12.5	0	20	20	0	0
Trimethoprim/ Sulfamethoxazole	25	0	20	40	0	0
Chloramphenicol	12.5	0	20	20	0	100
Nitrofurantoin	37.5	100	100	100	100	100

Out of 8 samples of *E. coli*, most of the isolates were resistant to ampicillin, ceftazidime and cefpodoxime, amoxicillin/clavulanate, cefotaxime, cefoxitin, ceftriaxone, and cefuroxime. Most of *E. coli* were sensitive to chloramphenicol, ciprofloxacin, ofloxacin, gentamicin, azithromycin, trimethoprim/sulfamethoxazole, and nitrofurantoin (Table 4 and Fig. 2). All the five isolates identified as *Enterobacter* spp were resistant to ampicillin, amoxicillin/clavulanate, cefotaxime, cefoxitin, ceftazidime, cefpodoxime and nitrofurantoin. Most of the strains were sensitive to chloramphenicol, ciprofloxacin, ofloxacin, trimethoprim/ sulfamethoxazole and gentamicin. All the five isolates of *Citrobacter* spp were sensitive to gentamicin; while were resistant to ampicillin, cefoxitin, nitrofurantoin. Most of the strains were resistant to ceftazidime, cefuroxime ceftriaxone, cefpodoxime, cefotaxime, and amoxicillin/clavulanate. Most of the *Citrobacter* spp were sensitive to chloramphenicol, ciprofloxacin, ofloxacin, and azithromycin. All the isolates identified as *Klebsiella* spp were resistant to ampicillin, amoxicillin/clavulanate, ceftazidime and nitrofurantoin; while were sensitive to cefotaxime, azithromycin, ofloxacin, trimethoprim/sulfamethoxazole, chloramphenicol and gentamicin. One-third of *Klebsiella* spp were resistant to ceftriaxone, cefoxitin, cefuroxime, cefpodoxime, and ciprofloxacin. *Shigella* spp (n=1) and *Yersinia* spp (n=1) were sensitive towards gentamicin, ofloxacin,

trimethoprim/sulfamethoxazole, azithromycin, and ciprofloxacin. The strains were resistant to ampicillin, amoxicillin/clavulanate, ceftazidime, cefpodoxime, and nitrofurantoin (Table 4).

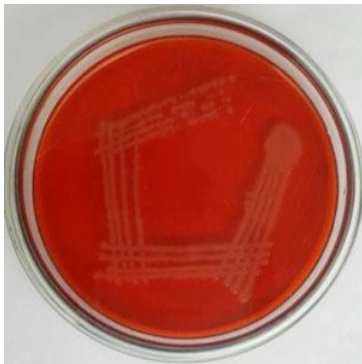


Fig 4. Biofilm non-former *Shigella* on Congo Red Agar Plate



Fig 5. Biofilm forming *Klebsiella* spp on Congo Red Agar Plate

Out of twenty-three isolates, 16 (69.6%) were found to be MDR. 100% of *Enterobacter* spp (n=5) and *Shigella* spp (n=1), 80% of *Citrobacter* spp (n=4), 62.5% of *E. coli* (n=5), 33.3% of *Klebsiella* spp (n=1) were multidrug resistant (MDR). Out of twenty-three isolated colonies, seven were found to be ESBLs producing. The percentage of ESBL producing *Enterobacteriaceae* was 60%, 40% and 25% of *Enterobacter* spp (EP S2, EP S6, EP S9), *Citrobacter* spp (EB S6, EB S8) and *E. coli* (EGM S7, EGM S8) respectively (Fig. 3).

Multiple antibiotic resistance (MAR) indices of bacteria revealed that none of the isolates were susceptible or resistant to all the fifteen tested drugs. Of all the twenty-three isolates, 18 had a MARI of greater than 20% giving an incidence of Multi-Antibiotic Resistance strains of 78.3%. Of all *Enterobacteriaceae*, 5 (21.7%) were resistant to 8 drugs (MARI = 0.53), 4 isolates (17.4%) were resistant to 10 drugs (MARI = 0.67), 4 (17.4%) were resistant to 9 drugs (MARI = 0.6), 4 (17.4%) were resistant to 5 drugs (MARI = 0.33), 2 (8.7%) were resistant to 4 drugs (MARI = 0.27). One isolate (4.3%) had a MARI of 0.13, 0.8, 0.87, and 0.93 each (Table 5).

All *Klebsiella* spp (Fig 5), *Enterobacter* spp, and *Yersinia* spp; 80% of *Citrobacter* spp; and 75% of *E. coli* were biofilm producing bacteria while *Shigella* spp was biofilm non-forming bacteria (Fig. 4). Although there is no significant relationship between the type of bacterial strains of *Enterobacteriaceae* and their response to the antibiotics at $df = 1$ and $p = 0.05$, the association between them is very strong with Cramer's V test value ranging between 0.724 and 1.

Table 5. Multiple antibiotic resistance (MAR) indices of *Enterobacteriaceae*

Resistant antibiotics	Sample	Strains	MARI
AMP + AMC + CX + CXM + CPD + CAZ + CTR + CTX + CIP + OF + GEN + COT + NIT + AZM	EPN S1	<i>Enterobacter</i>	0.93
AMP + AMC + CX + CXM + CPD + CAZ + CTR + NIT	EP S1	<i>Klebsiella</i>	0.53
AMP + AMC + CX + CXM + CPD + CAZ + CTX + COT + NIT + AZM	EBN S1	<i>E. coli</i>	0.67
AMP + AMC + CX + CXM + CPD + CAZ + CTR + CTX + NIT	EP S2	<i>Enterobacter</i>	0.6
AMP + AMC + CX + CXM + CPD + CTR + CTX + NIT	EPN S2	<i>E. coli</i>	0.53
AMP + CPD + CAZ + CTR + CTX	EPN S3	<i>E. coli</i>	0.33
AMP + CX + CXM + CPD + CAZ + CTR + CTX + CIP	EP S3	<i>Citrobacter</i>	0.53
AMP + AMC + CPD + CAZ + AZM	EGM S4	<i>E. coli</i>	0.33
AMP + AMC + CPD + CAZ + NIT	EB S4	<i>Yersinia</i>	0.33
AMP + AMC + CAZ + NIT	EPN S4	<i>Klebsiella</i>	0.27
AMP + CX + CXM + CPD + CAZ + CTR + CTX + CIP + OF + COT + NIT + AZM + C	EP S4	<i>Citrobacter</i>	0.87
AMP + CAZ	EGM S5	<i>E. coli</i>	0.13
AMP + AMC + CX + CXM + CPD + CAZ + CTR + CTX + COT + NIT	EB S6	<i>Citrobacter</i>	0.67
AMP + AMC + CX + CXM + CPD + CAZ + CTX + NIT + AZM	EP S6	<i>Enterobacter</i>	0.6
AMP + AMC + CX + NIT	EP S7	<i>Citrobacter</i>	0.27
AMP + AMC + CX + CXM + CPD + CAZ + CTR + CTX + CIP + OF + GEN + COT	EGM S7	<i>E. coli</i>	0.8
AMP + AMC + CX + CXM + CPD + CAZ + CTR + CTX + NIT	EB S8	<i>Citrobacter</i>	0.6
AMP + AMC + CX + CXM + CPD + CAZ + CTR + CTX	EGM S8	<i>E. coli</i>	0.53
AMP + AMC + CX + CXM + CPD + CAZ + CTR + CTX + NIT + C	EP S9	<i>Enterobacter</i>	0.67
AMP + AMC + CAZ + CIP + NIT	EM S9	<i>Klebsiella</i>	0.33
AMP + AMC + CX + CPD + CAZ + CTR + CTX + NIT	EM S10	<i>Enterobacter</i>	0.53
AMP + AMC + CX + CXM + CPD + CAZ + CTR + CTX + NIT	EGM S10	<i>E. coli</i>	0.6
AMP + AMC + CX + CXM + CPD + CAZ + CTR + CTX + NIT + C	EP S10	<i>Shigella</i>	0.67

AMP = Ampicillin, AMC = Amoxicillin/Clavlanate, CX = Cefoxitin, CXM = Cefuroxime, CTX = Cefotaxime, CAZ = Ceftazidime, CTR = Ceftriaxone, CPD = Cefpodoxime, GEN = Gentamicin, AZM = Azithromycin, CIP = Ciprofloxacin, OF = Ofloxacin, COT = Trimethoprim/sulfamethoxazole, C = Chloramphenicol, NIT = Nitrofurantoin.

Discussion

Presence of 30% *Klebsiella* spp in this study sample was higher than that of North Ethiopia (16.7%) (Asfaw et al., 2017). *E. coli* in 80% samples which was much higher than the Resende (2009). *Citrobacter* spp (50%) and *Enterobacter* spp (50%) were too high (17% and 7%, respectively) against the study of Tesfaye et al. (2019).

All the isolates of *E. coli* and *Klebsiella* were resistant to ampicillin which agreed with Asfaw et al. (2017). The present study showed *E. coli* showing resistant to ceftazidime (87.5%) and cefpodoxime (87.5%) which was higher than the findings of Korzeniewska et al. (2013) who reported 74.2% and 64.6% resistance to ceftazidime and cefpodoxime, respectively. The resistance for trimethoprim/sulfamethoxazole (12.5%) was nearly equal to the findings (26.6%) of Korzeniewska et al. (2013). In Brazil, Resende (2009) found all (n=8)

E. coli was sensitive to ceftazidime, cefotaxime, ceftriaxone, and cefpodoxime; while this study presented that 75% of *E. coli* were resistant to cefotaxime and 62.5% to ceftriaxone. Ciprofloxacin resistance (12.5%) was higher (20%) in Resende (2009). The present study showed that 12.5% of *E. coli* was resistant to gentamicin and chloramphenicol which differed from the study done in Poland (61.3% and 20.8%) by Korzeniewska et al. (2013). The findings of *Enterobacter* greatly varied with Asfaw et al. (2017).

All *Citrobacter* were sensitive to gentamicin, but 100% resistant to ampicillin, and 80% to ceftazidime, and ceftriaxone. Asfaw et al. (2017) found *Citrobacter* showing 100% resistance to ampicillin and 50% to gentamicin and ceftazidime. All *Klebsiella* were resistant to ampicillin and amoxicillin/clavulanate, while were sensitive to trimethoprim/sulfamethoxazole, chloramphenicol and gentamicin. Asfaw et al. (2017) showed resistance to gentamicin (57%), cotrimoxazole (57%), ciprofloxacin (50%), chloramphenicol (43%), ceftriaxone (64%) and amoxicillin/clavulanate (50%). One third of *Klebsiella* were resistant to ceftriaxone and ciprofloxacin. Only isolated strain of *Shigella* showed sensitivity towards gentamicin, trimethoprim/sulfamethoxazole, and ciprofloxacin. The strain was resistant to ampicillin, amoxicillin/clavulanate, ceftazidime, ceftriaxone. Asfaw et al. (2017) found *Shigella* showing 67% resistance to cotrimoxazole, ampicillin and ceftriaxone; while 33% resistance to amoxicillin/clavulanate, ceftazidime, ciprofloxacin, and gentamicin.

In this study 69.6% of isolates were found to be MDR which was slightly lower than North Ethiopia (76.2%) (Asfaw et al., 2017). Resistance to drugs may be due to the presence of the capsule, multidrug efflux pump, and greater efficiency to acquire and disseminate resistance plasmid. Resistance to class penicillin has been from the start of antibiotics and its usage over the decades have made the pathogen resistant to it (Mahato et al., 2018). So, the highest resistance to ampicillin can be understood. The increasing resistance to ceftazidime, ceftriaxone, cefotaxime, and cefoxitin may be due to the increasing clinical use of third generation cephalosporins.

The presence of 43.7% of ESBLs among total MDR was greater than 27.3% of Tesfaye et al. (2019). Twenty-five percent of ESBL-producing *E. coli* in this study was slightly lower than the study (37.1%) of Poland by Korzeniewska et al. (2013). Unregulated and haphazard exposure of antibiotics to bacteria provides the ability to develop resistance and produce enzymes that destroy the active sites of extended- spectrum third generation cephalosporins and monobactams thereby making bacteria resistant to such important antibiotics (Mahato et al., 2018).

On the basis of Multi-Antibiotics Resistance Index, 78.3% isolates were Multi-Antibiotics Resistance strains which was lower than the findings of Oli et al. (2017) who reported a MARI of 100%. This study showed higher number of *Klebsiella* spp, *Enterobacter* spp, *Citrobacter* spp, and *E. coli* were biofilm producing bacteria than the findings of Maheshwari et al. (2016). A biofilm is complex communities of surface associated cells enclosed in a polymer matrix which protects microorganisms from antimicrobial substance, opsonization, antibodies, phagocytosis and removal via the ciliary action of epithelial cell. So, biofilm producing property is a big threat to empirical treatment, if the infection occurs through such water (Maheshwari et al., 2016). This clearly indicates the need of sterilization of hospital wastewater to remove biofilm producing microbes.

Conclusion

The hospital effluent discharged into urban sewerage systems without adequate treatment is the major environmental concern of the day. The distribution of antibiotic resistant bacteria in the environment has been established through this study. Nearly 70% MDR bacteria and 44% ESBL producing bacteria in sewage water presents an alarming threat to such infection via contaminated food and water. Despite strong association, there is no significant relationship between the type of bacterial strains of *Enterobacteriaceae* and their response to the antibiotics. Hospital wastewater can negatively affect to the ecological balance and public health. Many hospitals in Nepal have no wastewater treatment facilities. Urgent measures are needed to minimize the effects from the release of hospital wastewaters into water resources. Sewage treatment plant must be established in hospital for their effluents and sludge coming from it.

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Conflict of interest

The authors declare that they have no competing interests.

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