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Abundance and phenotypic-genotypic analysis of antibiotic-resistant *Escherichia coli* isolated from wastewater of the Zainoel Abidin Hospital, Banda Aceh, Indonesia

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Abstract. *Anelia N, Suhartono S, Hayati Z. 2023. Abundance and phenotypic-genotypic analysis of antibiotic-resistant* Escherichia coli *isolated from wastewater of the Zainoel Abidin Hospital, Banda Aceh, Indonesia. Biodiversitas 24: 2513-2520.* Excessive and inappropriate use of antibiotics can initiate the emergence of antibiotic resistance in bacteria. Wastewater Treatment Plant (WWTP) is believed to be a reservoir for antibiotic-resistant bacteria, including *Escherichia coli.* This study aimed to perform the enumeration, isolation, characterization, and evaluation of resistance properties and antibiotic resistance-coding genes encoded by a plasmid of *E. coli* isolates from WWTP of the dr. Zainoel Abidin General Hospital (RSUDZA). The study was conducted through several stages, including the isolation and quantification of *E. coli*, antibiotic resistance testing, confirmation testing of ESBL-*E. coli* isolates, isolation of plasmid DNA, and amplification of TEM, SHV, and CTX-M genes. The results showed that the wastewater in the sum pit tank, aeration tank, and final tank of the WWTP contained *E. coli* at $7.5x10^4$ CFU/mL, $3x10^2$ CFU/mL, and $1x10^2$ CFU/mL, respectively. A total of 22 isolates were obtained, with ten isolates from the sum pit tank, seven from the aeration tank, and five from the final tank. *E. coli* isolates had resistance to eight types of antibiotics in the aeration tank and seven types in the final tank, compared to the sum pit tank of the WWTP. Therefore, 59% of the isolates were classified as Multi-drug Resistant (MDR) and 32% as Extended Spectrum β-Lactamase *E. coli* (ESBL-EC). TEM, SHV, and CTX-M genes were detected in all locations at 63.6%, 22.7%, and 95.5%, respectively. In addition, CTX-M was the most common gene found in wastewater isolates at RSUDZA WWTP. The hospital wastewater treatment process must be improved to reduce the presence of antibiotic-resistant bacteria and the spread of antibiotic resistance genes.

Keywords: Antibiotics, *Escherichia coli*, hospital, plasmid, resistance, WWTP

Abbreviations: ARB: Antibiotic-Resistant Bacteria, ESBL-EC: Extended Spectrum β-Lactamase *E. coli*, MDR: Multi-Drug Resistance, WWTP: Wastewater Treatment Plant, RSUDZA: Zainoel Abidin General Hospital

INTRODUCTION

Antibiotic resistance has become a major global threat generating significant implications in both clinical and nonclinical settings in recent decades. Resistance and the ability to spread resistance genes in bacteria occur in almost all classes of antibiotics, led by intensive and inappropriate uses (Ebmeyer et al. 2021). Antibiotic resistance can lead to many negative consequences, including more difficult and costly treatments, longer hospital stays, decreased effectiveness of antibiotics, increased toxicity, higher rates of illness, and even death (Friedman et al. 2016). In addition, antibiotic resistance can also have negative impacts on the environment. Potentially, the transfer of bacteria and genes results in mutations within populations of microbes, animals, and humans (Singer et al. 2016; Larsson and Flach 2022).

Among Antibiotic-Resistant Bacteria (ARB), *Escherichia coli* is among the most dangerous (Foudraine et al. 2021). Multidrug-resistant *E. coli* is included in the World Health Organization's (WHO) high-priority pathogens. That encourages the development of new antibiotics or other innovations to overcome the dissemination of this pathogen. Surveillance results from 2020 showed that antibiotic-resistant *E. coli* was present with a frequency of 541/2,782 (19.4%), 803/8,846 (9.1%), and 2,277/5,081 (44.8%) in blood, lower respiratory tract, and urine samples from 24 hospitals in Indonesia. Almost all *E. coli* isolated as the Extended Spectrum β-Lactamase (ESBL) (Anggraini et al. 2021).

Escherichia coli resistance to antibiotics persists even after treatment has been completed (Suhartono et al. 2018). Horizontal gene transfer mechanisms, such as plasmid-facilitated conjugation, cause resistance to be easily transferred through the environment through water, soil, food, air, and living organisms (Frieri et al. 2017). Hospitals are a source for developing and disseminating ARB in patients, staff, and even Wastewater Treatment Plants (WWTP) (Lien 2017); Hospital wastewater is a reservoir for ARB. According to the analysis of 134 isolates from WWTP Ayder Referral Hospital in North Ethiopia, 62.7% of bacterial isolates were detected in untreated hospital wastewater, and 37.3% were found after treatment in a WWTP (Asfaw et al. 2017). The number of

E. coli ESBL producers increases from 2 isolates in the influent to 5 in the effluent tank. ESBL encoding bla_{TEM}, $bla_{SHV,}$ and bla_{CTX-M} genes present at rates of 82%, 48%, and 67%, respectively (Chagas et al. 2011). The concentration of ARB increased in treated hospital wastewater with the gene obtained is blaTEM-1 (Yao et al. 2021). *E. coli* in treated wastewater becomes more resistant to several antibiotics. For example: in raw wastewater, the percentage of *E. coli* resistance to Meropenem, Ciprofloxacin, and cefixime were 3.8%, 53.8%, and 56.3%. In comparison, treated wastewater reached 20%, 60%, and 80% (Kristanto and Koven 2019).

Hospital discharges increase proportionally to the need for health services every year continuously. WWTP has been applied to hospitals in Aceh, yet not all of them function effectively or fulfill all standards (Kusuma et al. 2017). Effective wastewater treatment is necessary to prevent the spread of bacteria from hospital wastewater to the environment. While there has been significant research on treating hospital wastewater, there has been relatively little focus on the presence and characteristics of antibiotic-resistant bacteria or superbugs in Indonesia. Studying the antibiotic sensitivity and plasmid mediation of *E. coli* in hospital waste is important to understand the genetic factors contributing to resistance. This study aimed to identify, isolate, and characterize *E. coli* isolates recovered from the wastewater treatment plant of the Zainoel Abidin General Hospital (RSUDZA) and evaluate their antibiotic susceptibility and the presence of plasmid-mediated-antibiotic resistance genes.

MATERIALS AND METHODS

The collection of wastewater samples

Wastewater samples used in this study were collected with permission obtained ethics approval from the Health Research Ethics Committee (KEPK) Faculty of Medicine, Syiah Kuala University, RSUDZA (135/EA/FK-RSUDZA/2022); the flow of RSUDZA WWTP shown in Figure 1. The samples used were wastewater from WWTP contained in the sum pit tank, aeration tank, and final tank. Wastewater samples were collected using grab sampling methods following SNI 6989.59:2008. Wastewater from each tank was collected by immersing a 250 mL sterilized bottle (Schott) to a depth of around 5-10 cm until the bottle was filled. Bottles were labeled and stored in an ice box at 2-4°C and then transported to the laboratory immediately for further analysis.

Isolation, characterization, and quantification of *E. coli*

Isolation of *E. coli* was determined using the spread plate method. Six test tubes were filled with 9 mL of distilled water. Then, 1 mL of wastewater sample was added to the first series of dilutions, homogenized, and labeled as 10^{-1}. In the second series, a tube containing 9 mL of distilled water was inoculated with 1 mL of liquid from the 10^{-1} dilution and then homogenized. Multiple dilutions were performed up until a 10^{-6} dilution was obtained. Finally, 1 mL of the 10^{-6} dilution was inoculated onto Eosin Methylene Blue (EMB, Oxoid) agar medium, followed by incubation for 24 hours at 37°C (Asghari et al. 2021).

The growing isolates were then characterized using Gram staining and observed under a microscope at 1000X magnification. *E. coli* quantification was conducted by Total Plate Count (TPC) method using a 3 M Petri film *E. coli*/Coliform count plate (3 M Petri film). After inoculation, the 3 M Petri film *E. coli* plate was incubated at 37°C for 24 hours (Américo-Pinheiro et al. 2021).

Resistance analysis of *E. coli* isolates

Resistance assays of *E. coli* isolate to antibiotics were carried out by antibiotic disc diffusion method/Kirby Bauer, according to the standard Clinical Laboratory Standards Institute (CLSI 2022). First, pure bacterial culture was inoculated with an inoculation loop. Next, put into 5 mL of 0.85% NaCl and homogenized using a vortex (Biosan). Then, the suspension was adjusted to standard turbidity of 0.5 MacFarland. Next, a cotton swab was dipped into the suspension and spread onto plates containing Muller-Hinton agar (Oxoid). The following antimicrobial disk (Oxoid) were used: amoxicillin (25 µg), ampicillin (30 µg), penicillin (10 µg), nalidixic acid (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), gentamicin (10 µg), kanamycin (30 µg), streptomycin (10 µg), azithromycin (15 µg), tetracycline (30 µg), and chloramphenicol (30 µg) were placed on the plates. Finally, plates were incubated at 37°C for 24 hours (Suhartono et al. 2021).

ESBL isolate confirmation test

Confirmation of *E. coli* ESBL isolates was conducted by a double disk synergy test method. First, a 0.5 MacFarland *E. coli* suspension was evenly spread into Muller-Hinton agar (Oxoid). The latter was supplemented with third-generation cephalosporin antibiotic disks, cefotaxime (30 µg), ceftazidime (30 µg), and ceftriaxone (30 µg). Next, the three antibiotics were placed about 15 mm from amoxicillin-clavulanic acid (20:10 µg). Finally, plates were incubated at 37°C for 24 hours (Kaur et al. 2013).

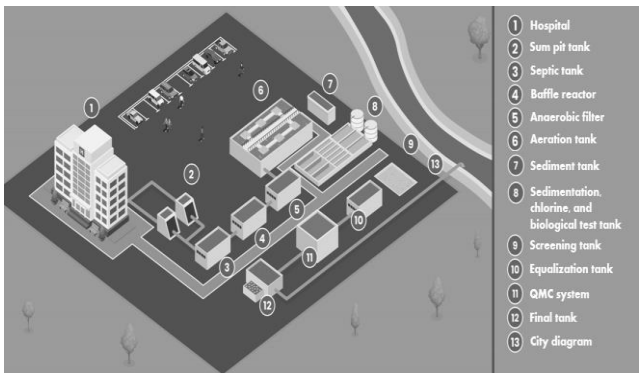

Figure 1. The schematic diagram of sampling sites of hospital WWTP of RSUDZA in Banda Aceh, Indonesia. The sum pit tank (No 2), aeration tank (No 6), and final tank (No. 12)

Table 1. PCR primers and cycle conditions

Primer	Primer sequence (5'-3')	Size (bp)	Reference	Cycle conditions
TEM (C) TEM (H)	5′- ATCAGCAATAAACCAGC -3′ 5′- CCCCGAAGAACGTTTTC -3′	516	Colom et al. (2003)	Initial denaturation at 95°C for 5 min 30 cycles of: Denaturation at 95°C for 30 s Annealing at 54,6°C for 1 min Extension at 72°C for 1 min Final extension at 72°C for 10 min
SHV (F) SHV (R)	5′- AGGATTGACTGCCTTTTTG-3’ 5′- ATTTGCTGATTTCGCTCG -3′	392	Colom et al. (2003)	Initial denaturation at 94°C for 5 min 35 cycles of: Denaturation at 94°C for 30 s Annealing at 51,7°C for 40 s Extension at 72°C for 40 s Final extension at 72°C for 10 min
CTX-M (F) CTX-M (R)	5’-TTTGCGATGTGCAGTACCAGTAA-3’ 5'- CGATATCGTTGGTGGTGCCATA -3’	544	Edelstein et al. (2003)	Initial denaturation at 94°C for 2 min 35 cycles of: Denaturation at 95°C for 20 s Annealing at 51,3°C for 30 s Extension at 72°C for 30 s Final extension at 72°C for 3 min

Plasmid DNA Isolation and Amplification of TEM, SHV, and CTX-M genes

Plasmid isolation was performed by culturing antibiotic-resistant *E. coli* isolates on LB media (Oxoid) and incubated on an orbital shaker incubator (Biosan) at 37°C for 24 hours. Once the bacterial suspension was ready for plasmid isolation, it was conducted using a ZymoPURETM Plasmid Miniprep kit (Zymoresearch, USA) according to the manufacturer's instructions. The extracted plasmid DNA was used as a template to determine the occurrences of antibiotic resistance genes of TEM, SHV, and CTX-M using PCR amplification (Garrec et al. 2011) with primer sets outlined in Table 1. All PCR amplifications were performed in 10 µL reactions containing 5 µL HotStarTaq master mix 1× (Meridian Bioscience), 0.4 µM of each primer (IDT), and 0.6 µL of template DNA. DEPC-treated water (EMD Millipore, Darmstadt, Germany) was used as No Template Control (NTC) run in parallel with samples. The PCR reactions were carried out using a PCR thermocycler (Sensquest) under conditions shown in Table 1. The PCR products were analyzed on 1 % (w/v) agarose gels (Invitrogen) at 100V for 24 min in 1X TBE buffer (HiMedia). In addition, agarose gels were stained with GelRed (Biotium) and visualized using GelDoc (Fire Reader) (Pavez et al. 2019).

Data analysis

The isolated bacteria from wastewater treatment were descriptively analyzed based on their phenotype and genotype. Data were tabulated using Microsoft Excel to display data in tables and figures. Furthermore, statistical analysis was performed using Prism9 and Statistical Package for Social Science (SPSS) version 26 with a p-value <0.05.

RESULTS AND DISCUSSION

Isolation, characterization, and quantification of *E. coli*

The results of *E. coli* quantification showed the presence of *E. coli* and coliform bacteria in the samples. The presence of *E. coli* bacteria is characterized by blue colonies with bubbles and coliforms by red colonies with bubbles. The mean number of *E. coli* colonies plotted on a logarithmic scale was 5.855 log CFU/mL (equivalent to $7.5x10^4$ CFU/mL) in the sum pit tank (influent), 2.217 log CFU/mL (equivalent to $3x10^2$ CFU/mL) in the aeration tank, and 1.669 log CFU/mL (equivalent to $1x10^2$ CFU/mL) in the final tank (effluent). In addition, the tanks followed by different letters (A and B) indicate that the mean cell density of *E. coli* in these tanks has a significant difference ($p<0.05$) with a value of $p=0.0002$ (Figure 2).

There were 22 *E. coli* isolates recovered in the three tanks, namely, ten isolates in the sum pit tank, seven isolates in the aeration tank, and five isolates in the final tank (Figure 3).

The isolates showed growth on selective media of EMB characterized by the formation of blackish-blue colonies with a metallic green sheen with various sizes of colonies (Figure 4). In microscopic observation, the isolates showed typical pink bacillus-shaped Gram-negative bacteria (Figure 5).

Antibiotic resistance and ESBL analysis of *E. coli* isolates

Regarding antibiotic susceptibility (Table 2), all 22 *E. coli* isolates from the three tanks resisted penicillin. Following penicillin, 50% of *E. coli* isolates in the sum pit were resistant to azithromycin. Furthermore, *E. coli* isolates in the aeration tank were resistant to amoxicillin, ampicillin, azithromycin, cefotaxime, and ceftriaxone at 57.14% of isolates. A total of 100% of *E. coli* isolates in the final tank were resistant to streptomycin, and 60% of isolates were resistant to azithromycin and ciprofloxacin.

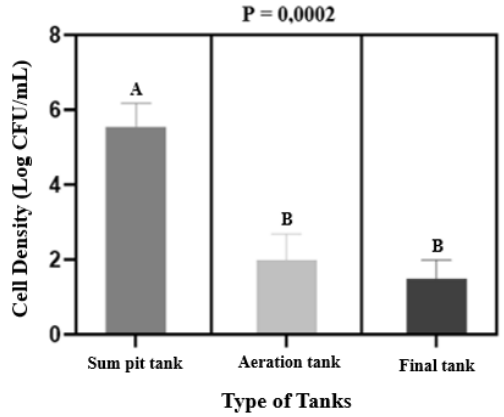

Figure 2. The means cell density in three tanks at WWTP of RSUDZA (Log CFU/mL). Different letters in the chart above indicate significant differences at the α=0.05 level

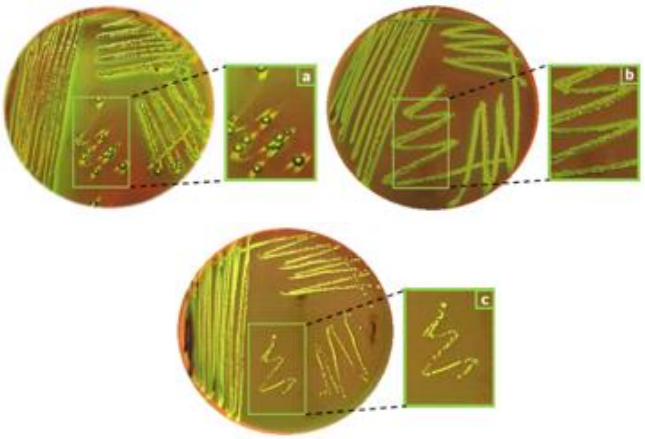

Figure 4. Colony morphology of *E. coli* isolates grown on the EMB media from the hospital WWTP, Banda Aceh, Indonesia, namely (A) sum pit tank; (B) aeration tank; and (C) final tank

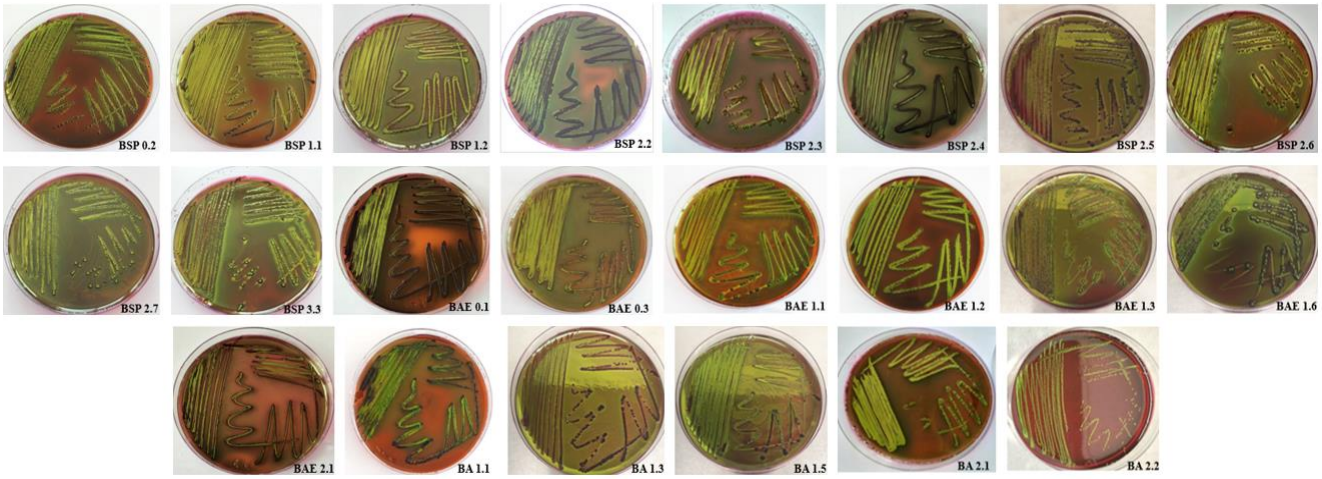

Figure 3. A total of 22 *E. coli* isolates were grown on the EMB media from the hospital WWTP, Banda Aceh, Indonesia. Namely, ten isolates in the sum pit tank (coded as BSP), seven isolates in the aeration tank (coded as BAE), and five isolates in the final tank (coded as BA)

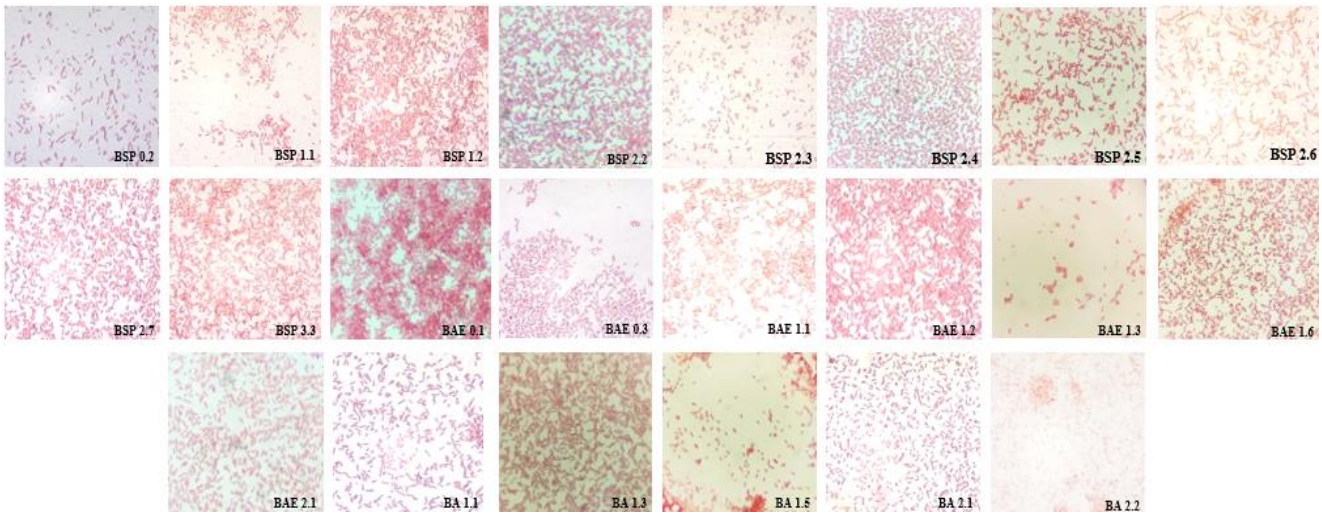

Figure 5. Gram staining of 22 *E. coli* isolates from hospital WWTP (1000x) Banda Aceh, Indonesia, namely ten isolates in the sum pit tank (coded as BSP), seven isolates in the aeration tank (coded as BAE), and five isolates in the final tank (coded as BA)

Table 2. Antibiotic resistance profiles of the 22 *E. coli* isolates collected from RSUDZA WWTP [a]

Antibiotic	Sum pit tank (n=10)			Aeration tank (n=7)			Final tank (n=5)		
	S	I	R	S	I	R	S	I	R
Amoxicillin	6 (60)	0 (0)	4 (40)	3 (42.9)	0 (0)	4 (57.1)	3 (60)	0 (0)	2 (40)
Ampicillin	4 (40)	2 (20)	4 (40)	3 (42.9)	0 (0)	4 (57.1)	3 (60)	0 (0)	2 (40)
Nalidixic acid	9 (90)	1 (10)	0 (0)	4 (57.1)	2 (28.6)	1 (14.3)	3 (60)	0 (0)	2 (40)
Azitrhomycin	5 (50)	0 (0)	5 (50)	2 (28.6)	1 (14.3)	4 (57.1)	2 (40)	0 (0)	3 (60)
Gentamycin	10 (100)	0 (0)	0 (0)	5 (71)	0 (0)	2 (28.6)	4 (80)	0 (0)	1 (20)
Kanamysin	0 (0)	10 (100)	0 (0)	2 (28.6)	5 (71)	0 (0)	1 (20)	2 (40)	2 (40)
Cloramphenicol	10 (100)	0 (0)	0 (0)	5 (71)	0 (0)	2 (28.6)	5 (100)	0 (0)	0 (0)
Ofloxsacin	10 (100)	0 (0)	0 (0)	6 (85.7)	0 (0)	1 (14.3)	4 (80)	0 (0)	1 (20)
Penicillin	0 (0)	0 (0)	10 (100)	0 (0)	0 (0)	7 (100)	0 (0)	0 (0)	5 (100)
Cefotaxime	7 (70)	1 (10)	2 (20)	3 (42.9)	0 (0)	4 (57.1)	4 (80)	0 (0)	1 (20)
Amoxicillin-clavulanic	8 (80)	2 (20)	0 (0)	3 (42.9)	1 (14.3)	3 (42.9)	4 (80)	0 (0)	1 (20)
Ceftriaxone	8 (80)	0 (0)	2 (20)	3 (42.9)	0 (0)	4 (57.1)	4 (80)	0 (0)	1 (20)
Ceftazidime	9 (90)	1 (10)	0 (0)	4 (57.1)	1 (14.3)	2 (28.6)	4 (80)	0 (0)	1 (20)
Ciprofloxacin	8 (80)	0 (0)	2 (20)	3 (42.9)	1 (14.3)	3 (42.9)	2 (40)	0 (0)	3 (60)
Streptomycin	0 (0)	6 (60)	4 (40)	1 (14.3)	3 (42.9)	3 (42.9)	0 (0)	0 (0)	5 (100)
Tetracycline	8 (80)	0 (0)	2 (20)	6 (85.7)	0 (0)	1 (14.3)	3 (60)	0 (0)	2 (40)

Note: [a] Data are shown as No (%), n: number of isolates, S: Sensitive, I: Intermediate, R: Resistant

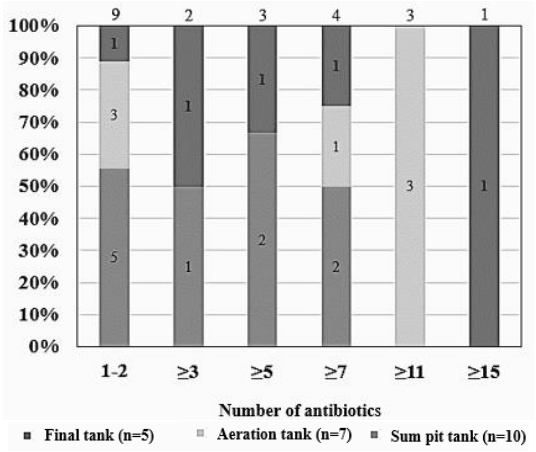

Figure 6. MDR *E. coli* isolates at hospital WWTP (n=22)

Table 3. Distribution of β-lactamase genotypes detected in *E. coli* isolates in hospital WWTP [a]

Gene	Sum pit tank (n=10)	Aeration tank (n=7)	Final tank (n=5)	Total (n=22)
TEM	3 (30)	7 (100)	4 (80)	14 (63,6)
SHV	1 (10)	2 (28,6)	2 (40)	5 (22,7)
CTX-M	9 (90)	7 (100)	5 (100)	21 (95,5)

Note: [a] Data are shown as No (%), n: number of isolates

Therefore, of the 22 isolates tested, nine (41%) were resistant to less than two antibiotics. In comparison, as many as 13 (59%) isolates have been resistant to more than three classes of antibiotics. That is known as Multi-Drug Resistance (MDR) (Figure 6).

Amplification of TEM, SHV, and CTX-M genes

Isolates in this study had β-lactam resistance genes in all three tanks. TEM, SHV, and CTX-M genes were detected in 63.6%, 22.7%, and 95.5% *E. coli* isolates, respectively (Table 3).

Discussion

The treatment process decreased bacterial cell densities in each tank, indicating that the WWTP at RSUDZA shows efficiency and effectiveness in reducing the number of *E. coli* cell densities. These findings align with those of previous studies. Wastewater treatment at the hospital WWTP in Northern Ethiopia reduced the number of *E. coli* isolates from 11 (in untreated wastewater) to 6 (in treated wastewater) and decreased the enumeration value from $4.5x10^5$ CFU/100 mL to $1.1x10^4$ CFU/100 mL (Asfaw et al. 2017). The treatment process at WWTP in the uMgungundlovu District demonstrated relatively high effectiveness in removing bacterial impurities. The influent water samples had a total *E. coli* count ranging from 6,5 × 10^4 to 10,8 × 10^4 CFU/mL, whereas the effluent water samples had a total *E. coli* count ranging from $1,8×10^3$ to $4,3×10^3$ CFU/mL (Gumede et al. 2021).

EMB media are useful to determine *E. coli* from other Gram-negative bacteria. It is easy to use and provides fast and inexpensive results. The metallic green color can appear less than six hours after the isolated bacteria (Leininger et al. 2001). *E. coli* colony isolates measure (2r) 2-3 mm, tend to be confluent and show a greenish metallic sheen (ThermoFisher 2022). The colony size formed varied in each tank. *E. coli* colonies in the sum pit tank tended to be larger than in the aeration and final tanks. This is caused by abundant organic nutrients in the sum pit tank compared to other tanks. Westfall and Levin (2017) nutrient availability directly correlates to *E. coli* cell size. Basavaraju and Gunashree (2022) *E. coli* are Gram-negative rod-shaped bacteria with a size of 1-3 µm × 0,4-0,7 µm, which we confirmed with Gram staining (see Figure 5).

Similar to the present study, resistance to β-lactam antibiotics, especially penicillin in *E. coli* from hospital WWTP, has been reported in several other studies. For example, *E. coli* isolates from hospital WWTP effluent in Slovak and Czech countries were 100% resistant to

ampicillin (Lépesová et al. 2020). Additionally, *E. coli* from hospital WWTPs in northwestern Ethiopia were 100% resistant to ampicillin (Moges et al. 2014).

Azithromycin is an antibiotic frequently found in WWTP effluent (Rodriguez-Mozaz et al. 2020). The Bushehr City (Iran), WWTP in two hospitals, seawater, and marine sediments have been polluted with azithromycin by 9, 6, 48, 3, and 4 times higher than the pre-COVID 19 eras caused by their high consumption of antimicrobial drugs in treating COVID-19 (Mirzaie et al. 2022). Azithromycin effectively performs as an alternative antibiotic in treating diarrhea caused by *E. coli* (Gomes et al. 2019; Tabrizi et al. 2022). The impermeable outer membrane of *E. coli* makes it naturally resistant to macrolides. But bacteria can still develop resistance by acquiring macrolide resistance genes through horizontal transfer. Azithromycin has been identified as the most potent macrolide against *E. coli*. Given the scarcity of new antimicrobials capable of fighting Gram-negative infections, the scientific community has shown interest in repurposing macrolides (Ma et al. 2022).

The Extended Spectrum β-Lactamase *E. coli* (ESBL-EC) isolate confirmation test is used in the identification of *E. coli* that has been resistant to third-generation cephalosporin antibiotics. In the sum pit tank, the percentage of *E. coli* resistant to cefotaxime at 20%, ceftriaxone at 20%, and ceftazidime at 0%. The percentage of resistant *E. coli* from the aeration tank to cefotaxime, ceftriaxone, and ceftazidime reached 57.1%, 57.1%, and 28.6%, respectively. The final tank of *E. coli* resistant percentage to cefotaxime, ceftriaxone, and ceftazidime was 20%. (Table 2). Of all *E. coli* isolates, 7 (32%) isolates were ESBL-EC. ESBL-EC isolates in the sum pit tank were two (20%) isolates, the aeration tank was four (57,1%) isolates, and the final tank was one (20%) isolate. The aeration tank had the highest number of ESBL-EC compared to the other tanks, possibly due to the presence of ESBL-EC bacteria in clinical patient samples. This occurrence could lead to the transmission of ESBL-EC in the environment. *E. coli* isolates in clinical samples at dr. Zainoel Abidin Hospital are almost entirely ESBL, which is 89% (Hayati et al. 2019). Then, the number of *E. coli* that produce ESBL is more than other bacteria in urine samples (Hayati et al. 2021). In addition, Hospital effluent is a major contributor to ESBL-EC in municipal wastewater (Bréchet et al. 2014; Azuma et al. 2022).

Antibiotic resistance profiles in the three tanks showed increased resistance in the aeration tank of eight antibiotics and the final tank of seven antibiotics compared to the sum pit tank (Table 2). That indicates the wastewater treatment process at the WWTP RSUDZA increases the number of antibiotic-resistant *E. coli* bacteria. Hospital wastewater, specially treated water, contributes to antibiotic-resistant bacteria as it is rich in bacteria resistant to more than one antibiotic (Bojar et al. 2021). In addition, hospital effluent is a source of resistant bacteria that can transfer their resistance genes to other bacteria (Lépesová et al. 2020).

The number of MDR isolates in the sum pit tank, aeration tank, and final tank are 5 (50%) isolates, 4 (57.1%) isolates, and 4 (80%) isolates, respectively. The final tank is the highest MDR producing. In addition, the final tank also found isolates resistant to 15 types of antibiotics, namely isolate BA 1.1. Several previous studies also reported research on the increase in MDR isolates in the effluent tank compared to the inffluent tank in WWPT. MDR *E. coli* found in the WWTP input was detected in as much as 67.5% and effluent at 72.9% (Che et al. 2019). Hospital WWTP effluent in the Slovak Republic and Czech Republic was MDR *E. coli* by 79% (Lépesová et al. 2020). The percentage of *E. coli* resistant to most antibiotics was higher in effluent than inffluent at hospital WWTPs (Kumar et al. 2020).

Genotype testing is needed to determine the presence of genes that cause resistance. Of the total 22 isolates, the CTX-M gene was the most prevalent gene found in 21 (95.5%) isolates, followed by the TEM gene detected in 14 (63.6%) isolates and the SHV gene in 5 (22.7%) isolates. In wastewater treatment in uMgungundlovu, South Africa, the ESBL TEM, SHV, and CTX-M genes were 57%, 27%, and 67%, respectively (Gumede et al. 2021).

Several previous studies also reported research on the predominant existence of the CTX-M gene. Of 41 *E. coli* isolates in clinical samples at Wahidin Sudirohusodo General Hospital Makassar, 82% had the ESBL-producing CTX-M gene (Pratama et al. 2019). *E. coli* isolates in clinical samples in Romanian hospitals almost entirely have the bla-CTX-M gene, namely 92.8% of isolates (Ghenea et al. 2022). There is a CTX-M gene of 95% in a hospital WWTP in Wisconsin, United States (Liedhegner et al. 2022).

Moreover, the SHV gene is the rarest gene found in this study. SHV gene was detected in five isolates: BSP 2.7, BAE 1.2, BAE 2.1, BA 1.1, and BA 1.3. Nzima et al. (2020) reported that the SHV gene was not found in isolates from wastewater treatment plants. Adegoke et al. (2020) found that SHV genes were also not found in isolates from WWTP. Compared to isolates detected with only one gene, isolates containing all three β-lactamase genes (TEM, SHV, and CTX-M) showed more antibiotic resistance in phenotype assays. That indicates the presence of resistance genes causes bacteria to become more resistant to many antibiotics or MDR. Nikaido (2009) stated MDR tends to be caused by the accumulation of genes on the R plasmid. Each of which encodes resistance to a particular antibiotic. Obayiuwana and Ibekwe (2020) state that the prevalence of antibiotic-resistance genes in wastewater contributes to bacterial resistance to many antibiotics. All β-lactamase genes tested in this study were found in isolates at the aeration tank as two isolates (BAE 1.2, BAE 2.1) and in the final tank as two isolates (BA 1.1 and BA 1.3). That indicates the wastewater treatment process at the WWTP RSUDZA is expected to increase the phenotype and genotype of bacterial resistance to antibiotics. Bréchet et al. (2014) found that waste treatment at WWTP caused an increase in ESBL-EC.

In addition, the high presence of resistance genes in hospital WWTPs is also initiated by the tendency of WWTPs to function as reservoirs, sedimentation, and processing of all types of liquids, both from living things such as human excretion and microorganisms and from

other chemical solutions. Coutu et al. (2013) and Kumari et al. (2020), the presence of hormone residues, antibiotics, antiseptics, analgesics, and patient excretions in large amounts of hospital WWTP can support the increase in bacterial resistance to antibiotics. Kaur et al. (2020), the compartment in the WWTP has a different proportion, concentration, and condition according to its function in the treatment. Stressor changes can act as a trigger for the bacterial community, causing bacteria to become more resistant. Foroughi et al. (2020) wastewater treatment technology is basically not designed to remove complex contaminants such as antibiotics. Therefore, increased residual levels of antibiotics of various classes and other stressors in WWTPs facilitate increased bacterial antibiotic resistance (Tabrizi et al. 2022).

Overall, this present study indicates a high resistance pattern and the ESBL-EC gene's presence in the wastewater treatment plant RSUDZA Banda Aceh, particularly in the aeration tanks and final tanks. That may pose potentially hazardous risks, such as transmitting resistance genes to the environment, animals, and people. Therefore, the wastewater treatment process of the hospital must be improved to reduce the presence of antibiotic-resistant bacteria and the spread of antibiotic-resistance genes, such as incorporating an ultraviolet light-based waste treatment process following monitoring the occurrence of antibiotic resistance bacteria.

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