Rapid detection of drug-resistant *Escherichia coli* by Vitek 2 compact system

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Sewage treatment facilities aim to reduce biological contaminants such as pathogenic bacteria, fungi, protozoa, and viruses in wastewaters before discharging them to the receiving water bodies. However, several studies have shown the persistence of these contaminants throughout the sewage treatment process. In this study, the Vitek 2 compact system was used to detect the presence of *Escherichia coli* in three sewage treatment facilities located in the Pietermaritzburg urban area (South Africa), and its susceptibility to antimicrobial agents. *E. coli* has been recognized as an important Gram-negative rod-shaped human pathogen. The effluent and influent samples were analysed to determine the fate of *E. coli* and its susceptibility to 17 antimicrobial agents. The system identified the presence of drug-resistant *E. coli* in all of the tested samples, with the highest susceptibility being to ampicillin (33%) and trimethoprim/sulfamethoxazole (27%). The Vitek 2 compact system is a quick and powerful tool to identify antimicrobial-resistant bacteria in effluents and monitoring by this system can be used to prevent the outbreak of waterborne diseases.

INTRODUCTION

Wastewater is defined as water that consists of stormwater runoff, industrial, domestic, or commercial sewage, or any mixture thereof (Naidoo and Olaniran, 2014). This wastewater contains contaminants such as nutrients, microorganisms, and organic matter, as well as contaminants of emerging concern such as pharmaceuticals and personal care products (Ginebreda et al., 2010). Wastewater treatment processes are therefore designed to either remove or reduce these contaminants to acceptable levels before the effluent is discharged to the receiving water body (Frigon et al., 2013). However, several studies have revealed the ability of most waterborne pathogens, such as *Escherichia coli*, to survive the chlorination method and thus be released into the environment (Jjemba et al., 2010; Ramirez-Castillo et al., 2015), where, if ingested, inhaled or encountered by susceptible individuals, it can cause waterborne diseases (Leclerc et al., 2002).

According to the World Health Organisation (WHO, 2018), about 844 million people lack safe potable water, with 159 million people dependent on surface waters. Contaminated water is linked to different types of infectious diseases, such as gastroenteritis, cholera, salmonellosis, typhoid, hepatitis and dysentery, with diarrhoea as the common symptom (Colvin et al., 2016). It is estimated that 842 000 people die each year from diarrhoea, of which 361 000 are children younger than the age of 5 years (WHO, 2018). Globally, these diseases are estimated to cause an economic loss of 12 billion USD per annum (Alhamlan et al., 2015).

Furthermore, several studies have revealed the inability of wastewater treatment processes to remove antibiotic-resistant genes (ARG) (Shejale et al., 2020; Nguyen et al., 2021). This is a concern since the nutrient-rich activated sludge has been shown to be a perfect environment for transfer of antibiotic-resistant genes between bacteria (Berendonk et al., 2015; Di Cesare et al., 2016; Hembach et al., 2017). Thus, this can lead to the development of antimicrobial-resistant bacteria which can be discharged into the aquatic environment. Recently, antimicrobial-resistant bacteria have also been detected in treated effluents (Alexander et al., 2020); this is posing as a new concern threatening the healthcare system of the 21st century. Between the year 2014 and 2016, over 1 million people has been reported to have died due to conditions in which antibiotic-resistant bacteria were implicated (Alexander et al 2020), and this has been projected to increase in the coming decades (Humphreys and Fleck 2016). Therefore, improving water quality is a global goal that needs immediate attention, of which surveillance programmes can be a starting point.

However, such surveillance programmes are usually lacking in developing countries, including South Africa. Molecular methods which are quick and accurate are critically required in detecting and tracing *E. coli* in animals, food, and water, to minimize the size and number of waterborne disease outbreaks in developing countries (Fratamico et al., 2016). Bacterial detection and antimicrobial sensitivity testing by Vitek 2 compact system have been successfully conducted in positive blood cultures (Bazzi et al., 2017). In this study, Vitek 2 compact system was used to detect the presence of *Escherichia coli (E. coli)* in three sewage treatment facilities located in the Pietermaritzburg urban area (South Africa) and its susceptibility to antimicrobial agents. However, usage of this technique in wastewater treatment plant samples has been minimal to date and its application requires more research, since it is a robust, reliable technique offering both microbial identification and AST. The results of this study revealed that Vitek 2 compact system is a quick and powerful tool to identify

antimicrobial-resistant bacteria in the wastewater treatment plant effluents, and monitoring by this method can be used to prevent the outbreak of waterborne diseases.

METHODS

Wastewater sample collection and processing

The wastewater samples were collected monthly from August to December 2020 from 3 wastewater treatment plants around Pietermaritzburg, KwaZulu-Natal. Plant A (29°36'04.9"S 30°25'44.9"E) was the largest of the three treatment plants and serves the Msunduzi Local Municipality, whereas Plant B (29°29'36.0"S 30°14'01.6"E) serves Howick town and Plant C (29°41'01.9"S 30°27'53.4"E) services part of the Ashburton area (Fig. 1). All these wastewater treatment plants use chlorination as the chemical process of deactivating the microorganisms. Samples were collected into 500 mL sterile plastic bottles containing sodium thiosulphate to neutralize chlorine in effluents. The temperature and pH values were measured on-site using a portable pH meter (Hach, S.A). A sample volume of 100 mL of influent and 200 mL of effluent was used for the determination of bacterial concentration via the membrane filtration method (0.45 μm, Millipore, U.S) and the filtrate was then stored in phosphate buffer saline (PBS) at 4°C for downstream experimentation.

Determination of BOD and COD in wastewater samples

Standard methods (Baird et al., 2012) were used to determine the biological oxygen demand (BOD) values. Briefly, the optical density (OD) values were measured before and after the incubation period (at 21°C for 5 days), and the difference gave the BOD values. For chemical oxygen demand (COD), the wastewater samples were digested (for 2 h at 148°C) in a solution of potassium dichromate, sulphuric acid, silver sulphate and mercuric sulphate contained in the COD tubes. Following the digestion period, the samples were allowed to cool at room temperature and the COD values were read using a photometer.

Enumeration of *E. coli*

A sample volume of 100 mL was used to estimate the number of *E. coli* cells, according to the manufacturer's instructions, using the Colilert Quanti-Tray/2000 system (IDDEX, USA). Following the incubation period (22 h at 37°C), the Quanti-Trays were examined under long wave (366 nm) ultraviolet (UV) light, and wells that turned yellow and fluoresced were identified as *E. coli* positive and selected for AST analysis. Efficiency of WWTPs in removing *E. coli* and percentage and log reductions were calculated using Eqs 1 and 2, respectively (Microchemlab, 2021).

$$\text{Percent reduction} = \frac{(A-B) \times 100}{A} \quad (1)$$

$$\text{log reduction} = \log_{10}\left(\frac{A}{B}\right) \quad (2)$$

where *A* denotes the number of viable microorganisms before treatment, and *B* is the number of viable microorganisms after treatment.

Antimicrobial susceptibility test (AST) with Vitek 2 compact system

The quanti-Tray/2000 were disinfected using 70% ethanol and yellow and fluorescing (*E. coli*) wells were selected and aseptically punctured using sterile tips and sub-cultured onto XM-G agar (Hyserve, Germany) for 20 h at 37°C. The presumptive *E. coli* colonies (blue or blue purple) were transferred onto nutrient agar (Oxoid, UK) and incubated at 37°C for 24 h. Following the incubation period, the bacterial isolates were emulsified in a 3 mL 0.45% saline solution to the density of 0.5 to 0.63 McFarland
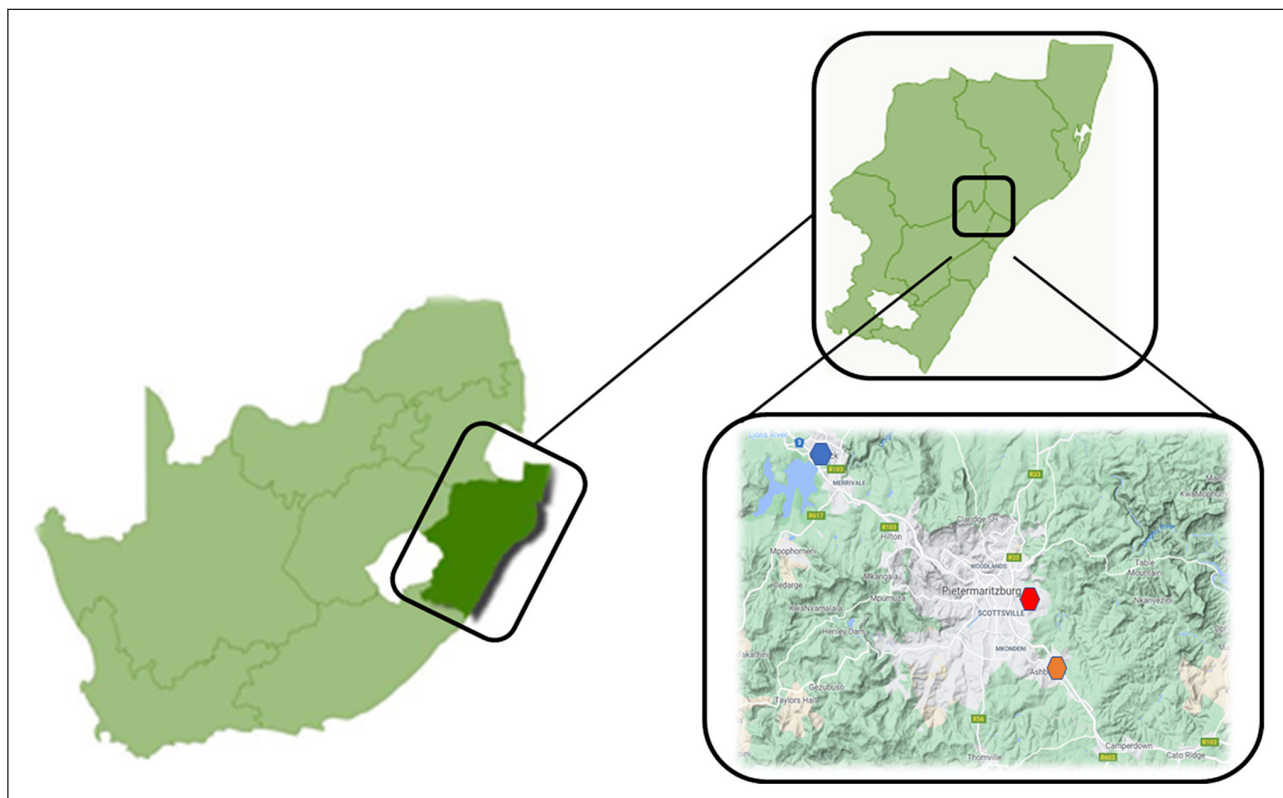

Figure 1. Location of the three South African wastewater treatment plants included in this study: WWTP1 – blue hexagon (29°36'04.9"S 30°25'44.9"E); WWTP:2 – red hexagon (29°29'36.0"S 30°14'01.6"E); WWTP3 – brown hexagon (29°41'01.9"S 30°27'53.4"E)

measurement using DensiCHEK Plus instrument (Biomerieux, USA). The prepared suspensions were used for both bacterial identification and AST using the Vitek 2 compact system (Biomerieux, USA) according to the manufacturer's instructions (Biomerieux, USA) – the Gram negative (GN) card was used for bacterial identification, and AST-N256 card was used for AST with Vitek 2 compact system. The selected *E. coli* isolates were tested against 17 antibiotics representing aminoglycoside, penicillin, carbapenems, cephalosporins, quinolones, tetracyclines and sulphonamides (Table 1). The AST-N256 card was automatically filled by a vacuum device, sealed, and inserted into the Vitek 2 reader-incubator module (incubation temperature: 35.5°C), and subjected to a kinetic fluorescence measurement for identification. Turbidity was measured as an indication of susceptibility every 15 min. At the completion of the incubation cycle, bacterial isolates were identified, and MIC values were determined for each antibiotic contained on the card. The *E. coli* ATCC 25922 was used as a positive control and the results were interpreted following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020).

Antimicrobial susceptibility testing using agar diffusion

The *E. coli* isolates were evenly spread onto the surface of nutrient agar and the disks were applied to the plate. After the incubation period (37°C for 20 h), the minimum inhibitory concentrations were recorded by determining the diameter of the inhibition zone using a ruler. The experiment was performed in duplicate.

Amplification of malate dehydrogenase (mdh) gene by colony PCR

Colony PCR was performed on the bacterial isolates for the molecular confirmation of isolated *E. coli* in a Bioer XP thermal cycler in a total reaction volume of 25 µL. Each reaction contained 12.5 µL 2 x Biolabs PCR master mix (containing DNA polymerase, PCR buffer and dNTPs), 2 µL 0.5 mM of the *mdh* forward (GGTATGGATCGTTCCGACCT) and reverse (GGCAGAATGGTAACACCAGAGT) primers (Omar and Barnard, 2010), 2 µL template DNA and PCR grade water to a volume of 25 µL. The *mdh* gene was amplified following modified Tarr et al. (2002) PCR conditions: initial denaturation step at 95°C for 15 min, followed by a 30-cycle reaction (consisting of denaturing at 95°C for 45 s, annealing at 65°C for 1 min 5 s, extension at 72°C for 2 min) and final elongation at 72°C for 5 min. The PCR amplicon was resolved on ethidium bromide–stained horizontal agarose gel (1.5 %, w/v) and electrophoresis was allowed to run for 1 h in electric field strength of 100 V using 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). The gel was visualized under UV light (BioRad, USA). The relative sizes of the DNA fragments were compared with molecular 100 bp marker run with the amplicons.

RESULTS AND DISCUSSION

Physicochemical properties of wastewater samples

BOD is a universal determinant of organic quality of water, and higher values (above 5 mgO$_2$/L) indicate poorer water quality (Lokman et al., 2020). In this study, the BOD values ranged from 85.6 to 283.0 mgO$_2$/L for the influent and 1.4 to 11.6 for the effluent while COD concentrations (mgO$_2$/L) ranged between 337 and 876 for the influent and 24.0 and 93.8 for the effluent over the period of 7 sampling events (Table 2). The highest pH value (8.0) was recorded in the influent of WWTP B while the temperatures (°C) ranged from 18.0 to 24.3 for the influent and 11.5 to 19.0 for the effluent. The BOD values (mgO$_2$/L) ranged from 29.6 to 208.0 for the influent and 1.4 to 11.6 for the effluent while COD concentrations (mgO$_2$/L) ranged between 658.9 and 1 023.0 for the influent and 20.8 and 66.8 for the effluent (Table 2). The pH values of wastewater samples at WWTP C ranged from 7.69 to 8.46 for the influent and 7.12 to 7.70 for the effluent while the temperatures (°C) ranged from 13.4 to 20.5 for the influent and 12.9 to 22.2 for the effluent. The BOD values (mgO$_2$/L) ranged from 93.4 to 258.0 for the influent and <1.0 to 6.4 for the effluent while COD concentrations (mgO$_2$/L) ranged between 354.0 and 791.0 for the influent and 44.3 and 97.8 for the effluent.

Table 1. Concentration range of antimicrobial agents used

Antibiotic groups	Antibiotics	Concentration range (µg/mL)
Aminoglycoside	Amikacin (AN)	8–64
	Gentamicin (GM)	4–32
	Tobramycin (TM)	8–64
Penicillin	Ampicillin (AM)	4–32
	Amoxicillin/clavulanic (AMC)	4–32/2–16
	Piperacillin/tazobactam (TZP)	2–48/4–8
Cephalosporin	Cefoxitin (FOX)	8–32
	Cefepime (FEP)	2–32
	Cefotaxime (CTX)	1–32
	Cefuroxime (CIP)	1–32
	Ceftazidime (CAZ)	2–32
Quinolones	Ciproflaxin (CIP)	0.5–4
Carbapenems	Ertapenem (ETP)	0.5–6
	Imipenem (IPM)	0.5–12
	Meropenem (MEM)	0.5–12
Tetracyclines	Tigecycline (TGC)	0.75–4
Sulphonamides	Trimethoprim/sulfamethoxazole (SXT)	1–16/19–304

Table 2. Physicochemical and microbiological parameters of WWTP A, WWTP B, and WWTP C

Sampling site	GPS coordinates	Sampling point	pH	BOD (mgO$_2$/L)	COD (mgO$_2$/L)
WWTP A	29°36′04.9″S 30°25′44.9″E	Influent	7.5	140.14	616.14
		Effluent	7.3	4.9	47.57
WWTP B	29°29′36.0″S 30°14′01.6″E	Influent	8.0	102.6	877.98
		Effluent	7.4	3.72	42.92
WWTP C	29°41′01.9″S 30°27′53.4″E	Influent	7.9	127.08	514.6
		Effluent	7.4	3.26	67.32
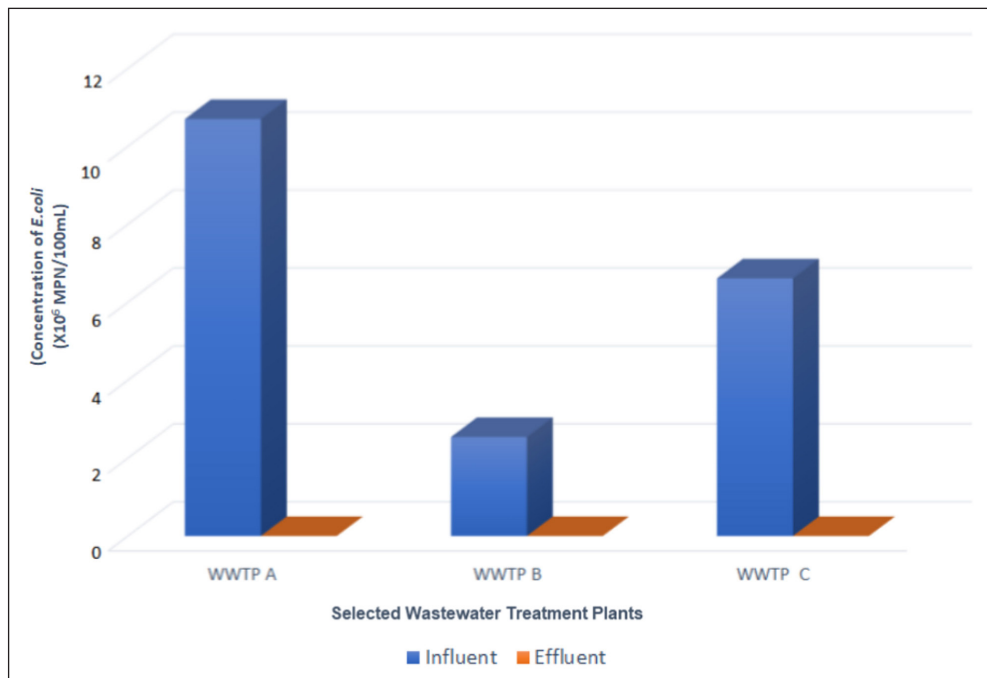
Figure 2. Performance of investigated wastewater treatment plants in removing *E. coli*

Log reduction of *E. coli* by wastewater treatment process

The concentration of *E. coli* in the influent collected from wastewater treatment process is shown in Fig. 2. WWTP A had a higher *E. coli* cell counts with a 4.6 log reduction, followed by WWTP B with a log reduction of 5.03 and WWTP C with a log reduction of 5.5. Taken together, it can be concluded that the three study sites had an average removal percentage of 99.999%. The results showed that the wastewater treatment processes employed within the chosen sampling sites are efficient in reducing the *E. coli* numbers; however, a concerning amount of *E. coli* cells persist throughout the treatment processes. A similar study by Brechet et al. (2014) revealed the release of a large number of *E. coli* in the treated effluent of Besancon city into the environment.

Identification of *E. coli* by Vitek 2 compact system

E. coli was positively identified in all tested samples, 59 from WWTP A, 36 from WWTP B and 40 from WWTP C using a Vitek 2 compact system. This was further confirmed by amplification of a 300 bp band fragment of *mdh* gene (Fig. A1, Appendix) on agarose gel, which corresponds to the presence of both commensal and pathogenic *E. coli* (Omar and Barnard, 2010). Furthermore, Anastasi et al. (2012) and Anastasi et al. (2013) have demonstrated the ability of pathogenic *E. coli* to survive the chlorination and UV stages of wastewater treatment plants. Therefore, new disinfection methods for complete inactivation of pathogenic microorganisms are urgently needed.

Antimicrobial-resistant *E. coli* identified by Vitek 2 compact system in wastewater

The ubiquity of antimicrobial-resistant *E. coli* was assessed for 17 antimicrobial agents (Table 2). As shown in Fig. 3, in WWTP A, the AM and SXT had > 20% resistance whereas the other antimicrobial agents showed between 0 and 5%. In WWTP B, the percentage resistance of AM and SXT was revealed as > 40%, whereas that of the others ranged between 0 and 15%. In WWTP C, both AM and SXT had the highest percentage resistance of > 40% and > 55%, respectively. The percentage resistance of the other antimicrobial agents ranged between 5% and 20%. It is interesting to note that AM and SXT experienced > 40% resistance in both WWTP B and WWTP C. furthermore, the data also showed WWTP C to be highly resistant to all the tested antimicrobial agents compared to WWTP A and WWTP B. Taken together, the data showed the highest prevalence of resistance in WWTP C, WWTP B and WWTP A to AM and SXT antimicrobial agents. This was also confirmed by the disk diffusion method (Fig. A2, Appendix). Ampicillin is a common antibiotic used for the treatment of *E. coli* infection in humans and livestock, and thus has a higher chance of selecting for resistant bacteria in the gut, including *E. coli*. According to the National Department of Health (2018), a quarter of antibiotics used to treat *Pneumonocystis jiroveci* in HIV patients in South Africa composed of trimethoprim, a constituent of co-trimoxazole (SXT). This high usage can likely induce resistance to gut microorganisms including *E. coli*. Contrastingly, our results

also showed 100% susceptibility towards carbapenems, amikacin, and tigecycline. This was to be expected since such antibiotics are being categorised as watch-and-reserve antibiotics by the National Department of Health (2018), and thus are sparingly used.

Furthermore, the efficiency of the targeted wastewater treatment plants in the removal of antibiotic resistant *E. coli* was also studied. The data revealed the complete elimination of *E. coli* resistant to FOX, TZP and AMC (Fig. 4). However, resistance to AM and SXT was still present in the effluent. Reinthaler et al. (2003) and Rodriquez et al. (2019) have shown the contribution of sewage treatment plants in the dissemination of antibiotic-resistant microorganisms in the environment.

In conclusion, the results of this study have revealed the potential use of the Vitek 2 compact system in monitoring wastewater samples for the presence of antimicrobial resistance *E. coil*. This method is not only confined to the detection of *E. coli* but can be used in the identification of other waterborne pathogens including *Salmonella* and *Vibrio cholerae* in wastewater samples. Since water released into the aquatic environment can be used for human activities including swimming, and fishing, understanding
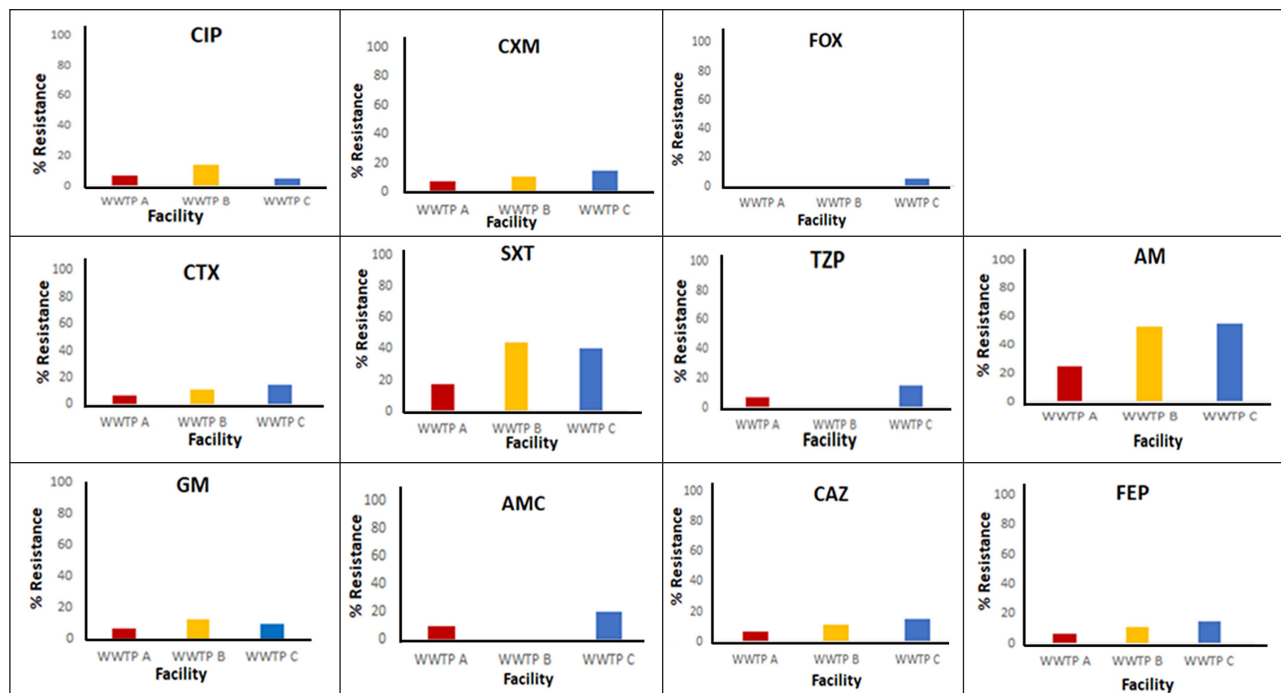

Figure 3. Percentage of antimicrobial resistance profiles in the selected wastewater treatment plants. Number of isolates from WWTP A (*n* = 59), WWTP B (*n* = 36), WWTP C (*n* = 40).
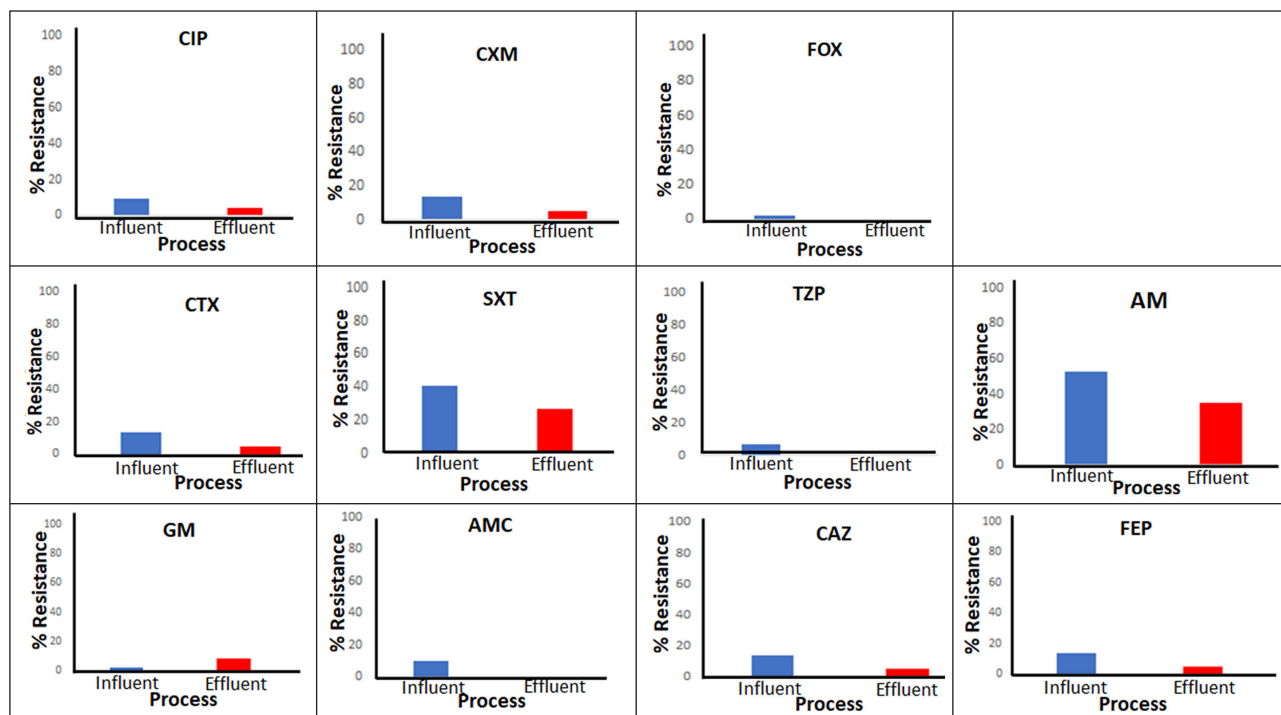

Figure 4. Removal profiles (%) of antimicrobial resistant *E. coli*. Number of isolates from WWTP A (*n* = 59), WWTP B (*n* = 36), WWTP C (*n* = 40)

the nature of pathogens released is important in order to prevent waterborne disease outbreaks.

ACKNOWLEDGEMENTS

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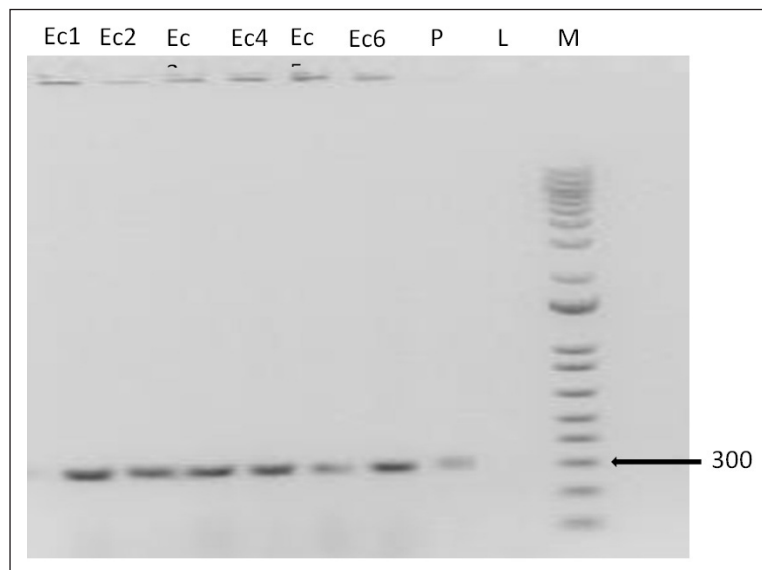
Figure A1. Horizontal gel electrophoresis shows the *mdh* gene fragments of *E. coli*, Ec (1–6): *E. coli*, M: DNA ladder
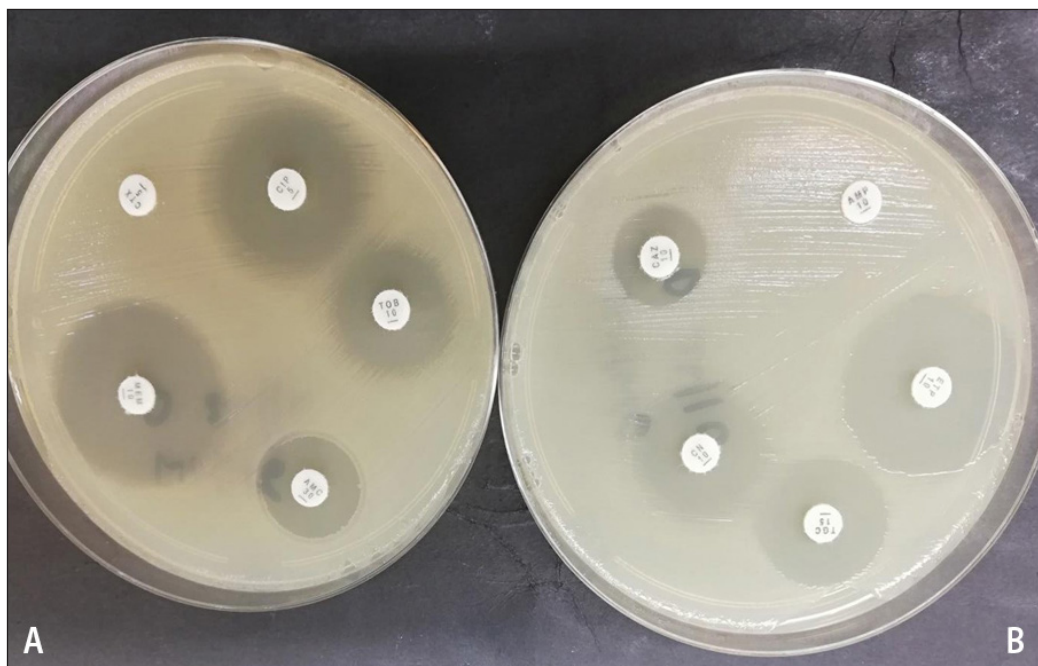

Figure A2. Disc diffusion method with zones of inhibition for CIP (diameters), TOB, AMC, CN, TOC, ETP and NEM against *E. coli*