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Research paper

Occurrence of multidrug-resistant *Escherichia coli* and antibiotic resistance genes in a wastewater treatment plant and its associated river water in Harare, Zimbabwe

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Wastewater treatment plants (WWTPs) have been identified as point sources of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARG). Due to variations in antibiotic use and prescribing patterns in different countries, it is imperative to establish the presence of ARB and ARGs in water environments on a country-by-country basis. This study investigated the occurrence of 11 antibiotic-resistance genes (*QNRB, DFR14, CTX-M, KPC, Sul1, QNRA, Sul2, ERMB, ERMA, SHV, NDM*), and antibiotic-resistant *Escherichia coli* in a WWTP and its associated river water in Harare, Zimbabwe. 24 water samples were collected across 3 sites: upstream and downstream of the WWTP; final effluent of the WWTP. The samples were collected weekly for 8 weeks. Pure cultures of the *E. coli* isolates were obtained by membrane filtration (0.45 µm) and repeated streaking on Tryptone Bile X-glucuronide followed by biochemical tests (indole test; citrate test; motility, indole, and ornithine). Antibiotic resistance profiling was done for 12 antibiotics using the disc diffusion method. Total genomic DNA was extracted from the 21 water samples and the occurrence of 11 antibiotic-resistant genes investigated using conventional PCR. 86 *E. coli* isolates were obtained from the sampled sites: 28 from the upstream site, 26 from the WWTP effluent, and 32 from the downstream site. The results from chi-squared analysis showed a significant association ($p < 0.05$) between the sampling site and the percentage of antibiotic-resistant *E. coli* for all 12 antibiotics investigated. The percentage of *E. coli* isolates resistant to the tested antibiotics varied from 29% (ertapenem) to 80.2% (ciprofloxacin). 81 (94.2%) *E. coli* isolates were resistant to antibiotics from ≥3 classes. Eight (8/11, 72.7%) ARGs were detected in the WWTP effluent and river water samples. Results indicate that the investigated WWTP and associated river water are reservoirs of ARGs and antibiotic-resistant *E. coli*, which is a public health concern.

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INTRODUCTION

Antibiotic resistance has become a global threat to public health systems around the world, as bacteria have developed various resistance mechanisms to antibiotics (De Kraker et al., 2016). Based on data from 204 countries, antibiotic resistance is currently estimated to be responsible for 1.27 million fatalities per year (Murray et al., 2022). Antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) have been detected and quantified from different environmentally relevant matrices, including WWTPs which are now considered hotspots for the development and dissemination of ARGs (Adefisoye and Okoh, 2016).

Wastewater reclamation facilities serve as reservoirs for antibiotic-resistance genes associated with human and animal pathogens and conventional wastewater treatment methods are unable to remove antibiotics from final effluent (Adefisoye and Okoh, 2016; Hirsch et al., 1999; Rather et al., 2017). WWTPs and surface water are repositories of multidrug-resistant *E. coli* isolates that harbour extended-spectrum β-lactamase (ESBL) genes (Nzima et al., 2020). In Zimbabwe, sewage and water treatment plants are not well developed and there is sometimes no final disinfection step in wastewater treatment (Nhapi et al., 2004). The catchment of the WWTP investigated in this study mainly comprises the watershed of the Mukuvisi River to the south and south east of downtown Harare. After biological nutrient removal, the wastewater from the WWTP is discharged into the river without any tertiary treatment.

Escherichia coli is a commensal bacterium that can be easily transmitted to the environment through manure, animal faeces, improperly treated wastewater or sewage, and sewage overflow caused by heavy rains (Mascher et al., 2017). *E. coli* can exist as a commensal in the intestinal flora of humans and animals or as a pathogen causing a range of infections. Most *E. coli* strains are excreted into the environment and many of them can produce ESBL enzymes (Kraemer et al., 2019; Mascher et al., 2017; Nzima et al., 2020). *E. coli* is readily found in water environments as a contaminant, and has thus been routinely used to study antibiotic resistance. *E. coli* is an excellent surrogate for investigating the spread of antibiotic resistance as it can easily acquire and transmit ARGs (Poirel et al., 2018).

Long-term monitoring of ARB in areas with treated or untreated sewage may provide an indication of the levels and development of local clinical resistance and reveal the ARB circulating within a given population (Kwak et al., 2015). However, information on the occurrence and prevalence of antibiotic-resistant *E. coli* and associated ARGs in wastewater effluent from WWTPs and associated river water in Zimbabwe is limited. This study, therefore, aimed to detect and characterise antibiotic-resistant *E. coli* and ARG genes in a wastewater treatment plant and its associated river water in Harare, Zimbabwe.

MATERIALS AND METHODS

Study site and sample collection

The WWTP catchment mainly comprises the watershed of the Mukuvisi River to the south and south east of downtown Harare. The solids (sludge) and liquid (effluent) portions of the incoming raw sewage are separated in the settling tank. The effluent overflows a circular weir and gravitates to the biological nutrient removal (BNR) unit. There is no chlorination done as the final treatment but rather the final effluent is discharged into Mukuvisi River after BNR treatment.

Sampling was done once a week from August to September 2021; thus a total of 8 samples were collected per site over a 2-month period. For each sampling event, 3 composite samples were collected upstream (Site 1), WWTP final effluent (Site 2), and downstream (Site 3) as shown in Fig. 1. Close to the downstream sampling site, there is an informal settlement. Field observations during sampling showed that the water at the downstream sampling site is used directly by the inhabitants of the informal settlement for several household purposes, including brushing teeth, bathing and washing, without any prior treatment. A total of 24 samples were collected, thus 8 samples per site over 2 months. All samples were collected using a Buerkle long handle sampler telescoop (QTE technologies, Vietnam) and aseptically transferred into 1 000 mL sterile plastic bottles (Microspec. UK/Bromborough). The samples were transported to the Water Quality Assurance Laboratory on ice to maintain a cool chain for immediate processing.

Isolation of *E. coli*

Ten-fold serial dilutions were done for all the samples and 100 mL was filtered. Membrane filtration was done by filtering 100 mL of each sample dilution through a 0.45 µm membrane filter using a membrane filtration unit. The filter papers were placed on Petri dishes containing TBX media (Oxoid, England/Hampshire). The Petri dishes were then incubated at 37°C for 24 h. Each dilution was replicated twice. Given the possibility that a large proportion of samples may contain many individual and often unrelated isolates, a random subset of 5 individual presumptive *E. coli* isolates was selected from each sample. These presumptive *E. coli* isolates were later confirmed using biochemical tests, including the indole test, citrate test and motility, indole, and decarboxylase test.

Molecular confirmation of *E. coli*

DNA extraction was done using a standard heat lysis protocol (Mbanga et al., 2018). *E. coli* was confirmed using conventional PCR of the *uid*A (β-D glucuronidase) gene. The PCR reaction consisted of a total reaction mixture of 10 uL with 5 µL of 2x Dreamtaq master mix (New England Biolabs, UK), 0.16 µL (0.4 µM) forward primer, 0.16 µL (0.4 µM) reverse primer (Inqaba Biotech, Pretoria, SA), and 2.68 µL nuclease-free water (New England Biolabs, UK), and 2 µL of DNA template was prepared and run on a MiniAmp Plus Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, USA). The negative control comprised nuclease-free water in place of template DNA. The PCR profile was as follows: initial denaturation at 95°C for 3 min, 30 cycles (denaturation at 95°C for 30 s, annealing temperatures at 50°C for 30 s, extension at 72°C for 1 min) and then the final extension at 72°C for 5 min. *E. coli* ATCC 25922 was used as a positive control.

Antibiotic susceptibility testing

Twelve commercial antibiotic discs (Mast Diagnostics, Merseyside, UK) were employed for the antibiotic susceptibility testing. This was done on a bacterial suspension with the same turbidity as the 0.5 MacFarland standard, which was used for the antibiotic susceptibility testing on Mueller Hinton agar. The following antibiotics were used: cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), cefepime (CPM, 30 µg), ceftriaxone (CRO, 30 µg), azithromycin (ATH, 15 µg), ampicillin (AP, 10 µg), amoxicillin/clavulanic acid (AUG, 20 µg/10 µg), ertapenem (ETP, 10 µg), trimethoprim/sulfamethoxazole (TS, 1.25 µg/23.75 µg), tetracycline (TET, 30 µg), nalidixic acid (NA, 30 µg), and ciprofloxacin (CIP, 5 µg). The antibiotic susceptibility testing and interpretation of results was determined using Clinical and Laboratory Standards Institute (CLSI) guidelines and interpretative charts (CLSI, 2020). *E. coli* ATCC 25922 was used for quality control. Multi-drug resistance (MDR) was defined as non-susceptibility to 1 or more antibiotics in 3 or more drug classes.

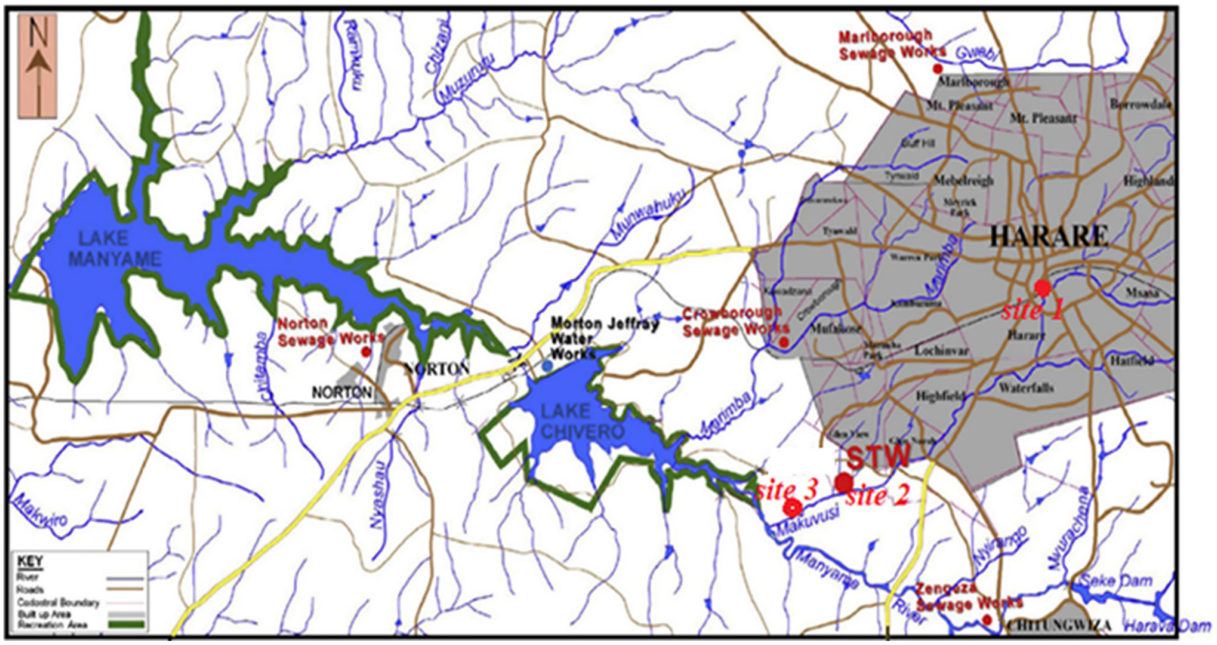

Figure 1. Map showing the location of the sampling sites: upstream (Site 1), the major wastewater treatment plant (Site 2) and downstream of the treatment plant (Site 3) (adopted from Muserere et al., 2014)

Extraction of total genomic DNA

Total genomic DNA was extracted from the 21 wastewater samples (7 from WWTP effluent, 7 from the upstream site, and 7 from the downstream site) using the Qiagen Blood and Tissue Kit (Qiagen, Manchester, UK) according to the manufacturer's protocol with few modifications. Samples collected in Week 1 were not available and hence were excluded. The samples were first centrifuged to get a pellet, and more of the sample was added at the beginning of each subsequent centrifuging round. This was done 5 times for each sample at 12 000 r/min for 5 min. DNA was then extracted from the sample following the manufacturer's instructions. The quality of the extracted DNA was checked using agarose gel electrophoresis.

Detection of antibiotic-resistance genes in the wastewater samples

After DNA was successfully isolated directly from the 21 samples, the occurrence of antibiotic-resistance genes was investigated using conventional PCR with specific oligonucleotide primers. The ARG primers used in this study are shown in Table A1 (Appendix). A total of 11 ARGs cutting across different classes of antibiotics (β-lactams, quinolones, macrolides, trimethoprim, and sulfonamides) were assayed.

Statistical analysis

The generated data were analysed in Microsoft Excel 2018 using STATA version 17 (Stata, 2017), using tab command (to create contingency tables) to check the prevalence of resistant isolates across the three sampling sites. The prevalence of resistance for all the isolates against a panel of 12 antibiotics was recorded from the STATA outputs. To check if there was an association between the sampling site and the prevalence of resistant isolates, the chi2 command was used in STATA version 17 and this was tested at a 5% significance level, testing the null hypothesis that there was no association between the sampling site and prevalence of multidrug-resistant *E. coli*.

RESULTS

Isolation and identification of *E. coli*

A total of 86 *E. coli* isolates were confirmed using biochemical and molecular techniques from the 24 wastewater samples. In total, 28 isolates were from the upstream site, 26 from the final effluent of the WWTP, and 32 from the downstream site.

Prevalence of antibiotic-resistant *E. coli*

The antibiotic susceptibility of the tested *E. coli* isolates across all sampled sites is shown in Fig. 2. The highest resistance was observed towards TS (80%) and CIP (80.2%), followed by TET (79%). *E. coli* isolates were mostly susceptible to the carbapenem ETP (29%) (Fig 2). Resistance to third-generation cephalosporins was found to be 37.2%, 39.5%, 40.6%, and 41.8% for the antibiotics cefepime, ceftazidime, cefotaxime, and ceftriaxone, respectively. Macrolide resistance (azithromycin) was also observed in 63.9% of the *E. coli* isolates. A higher prevalence of antibiotic resistance was recorded in *E. coli* isolates from the samples that were collected downstream for all the antibiotics (Table 1). The prevalence of antibiotic-resistant isolates from the upstream samples ranged from 21% (ceftriaxone) to 57%, which was jointly recorded for tetracycline and augmentin (AUG). For the final effluent isolates,

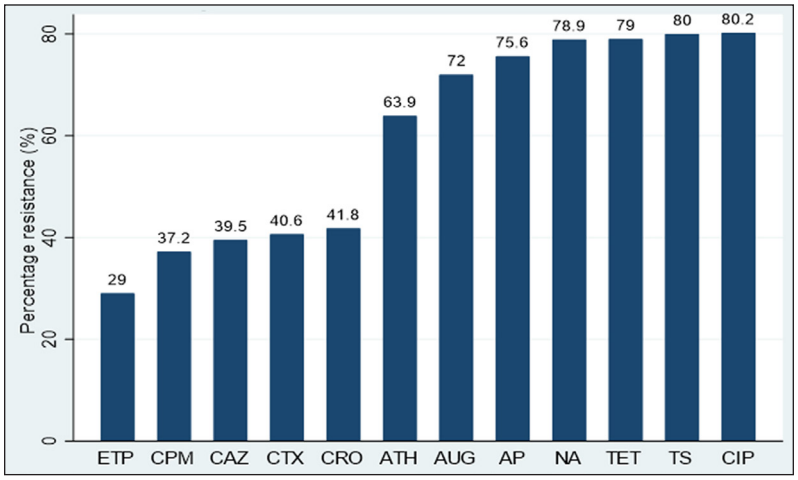

Figure 2. Percentage resistance of *E. coli* (*n* = 86) to different antibiotics. CTX = cefotaxime, CAZ = ceptazidime, CPM = cefepime, CRO = ceftriaxone, ATH = azithromycin, AP = ampicillin, AUG = amoxicillin clavulanic acid, ETP = ertapenem, TS = trimethoprim/sulfamethoxazole, NA = nalidixic acid, CIP = ciprofloxacin

Table 1. Prevalence of antibiotic resistance to studied antibiotics in *Escherichia coli* isolates for each sampling site

Sampling site	Macrolide	Cephalosporins				Penicillins		Carbapenems	Fluoroquinolone/ quinolones		Tetracyclines	Sulfonamides
	ATH N (%)	CTX N (%)	CAZ N (%)	CPM N (%)	CRO N (%)	AP N (%)	AUG *N* (%)	ETP *N* (%)	CIP *N* (%)	NA *N* (%)	TET *N* (%)	TS *N* (%)
Upstream (*n* = 28)	9 (32)	10 (36)	7 (25)	8 (29)	6 (21)	14 (50)	16 (57)	7 (25)	13 (46)	14 (50)	16 (57)	15 (54)
Effluent (*n* = 26)	18 (69)	12 (43)	12 (46)	11 (39)	9 (32)	21 (80)	21 (81)	8 (29)	25 (96)	22 (85)	21 (81)	23 (88)
Downstream (*n* = 32)	28 (88)	13 (46)	15 (47)	13 (41)	17 (53)	30 (93)	31 (97)	10 (36)	31 (97)	31 (97)	31 (97)	31 (97)

CTX = cefotaxime, CAZ = ceptazidime, CPM = cefepime, CRO = ceftriaxone, ATH = azithromycin, AP = ampicillin, AUG = amoxicillin clavulanic acid, ETP = ertapenem, TS = trimethoprim/sulfamethoxazole, NA = nalidixic acid, CIP = ciprofloxacin

the highest (96%) frequency of antibiotic-resistant isolates was recorded for CIP, and the lowest was observed for the antibiotic ETP (32%). However, for the downstream isolates, a relatively higher prevalence of antibiotic-resistant isolates was observed compared to other sites, with the highest prevalence (97%) being recorded for the antibiotics CIP, NA, TET, AUG, and TS (Table 1). The results from chi-squared analysis showed a significant association ($p < 0.05$) between the sampling site and antibiotic resistance for all 12 antibiotics at a 5% significance level. Therefore, the sampling site had an effect on the prevalence of antibiotic resistance among the isolates, with the downstream site recording the highest occurrence of antibiotic resistance for 11 of the 12 antibiotics that were tested (Table 1).

Prevalence of multidrug-resistant profiles

Out of the 86 isolates that were investigated for multidrug resistance, 81 (94.2%) were resistant to 3 or more antibiotics from different classes. Only 6 isolates from the upstream site were resistant to 2 or fewer antibiotics. However, all isolates were resistant to at least one antibiotic. A total of 48 multidrug-resistance patterns were observed from the 81 MDR *E. coli* isolates. The most prevalent MDR patterns were AUG-TET-ATH-CRO-CPM-CTX-CAZ-CIP-NA-AP-TS and AUG-TET-ATH-CIP-NA-AP-TS that were observed for 10 isolates each (Table 2). An isolate from the downstream site was resistant to all 12 antibiotics (Table 2).

Detection of antibiotic-resistance genes

Antibiotic-resistance genes were detected directly from 21 wastewater samples. A total of 8 (72.7%) different ARGs were detected across the sampled sites (Table 3). The distribution of the ARGs ranged between 0 and 81%. Most samples were found to harbour antibiotic-resistance genes (Table 3) for 2 or more classes of antibiotics. The *sul2* gene (81%) was the most prevalent ARG occurring at all 3 sampled sites. The *ermA*, *ermB*, *qnrA*, *qnrB*, and *CTX-M* genes also occurred at all 3 sites but at lower frequencies. The *NDM* gene was only detected in the final effluent of the WWTP (Table 3). The *KPC*, *SHV*, and *Dfr14* genes were not detected in any of the assayed water samples.

Table 2. Selected predominant multiple-antibiotic-resistant (MAR) phenotypes of *E. coli*. Some were common to different sampling sites.

MAR phenotype	Number observed (*n*)		
	Upstream	Effluent	Downstream
AUG-TET-ATH-CRO-CPM-CTX-CAZ-CIP-NA-AP-TS	1	0	9
AUG-TET-ATH-CIP-NA-AP-TS	0	3	7
AUG-TET-ATH-CIP-NA-AP-ETP-TS	0	1	5
AUG-TET-CIP-NA-AP-TS	0	1	2
AUG-CAZ-TS	3	0	0
TET-CIP-NA	2	0	0
TET-CAZ-CIP-NA	2	0	0
AUG-TET-ATH-CRO-CPM-CTX-CIP-NA-AP-TS	0	3	0
AUG-TET-ATH-CRO-CPM-CTX-CIP-AP	0	2	0
AUG-TET-ATH-CRO-CPM-CTX-CIP-NA-AP-ETP-TS	0	2	0
TET-NA-TS	0	2	0
AUG-TET-ATH-CRO-CAZ-CIP-NA-AP-TS	0	0	2
AUG-TET-ATH-CRO-CPM-CTX-CAZ-CIP-NA-AP-ETP-TS	0	0	1
AUG-TET-ATH-CRO-CPM-CTX-CAZ-CIP-NA-TS	0	0	1
AUG-ATH-CRO-CPM-CAZ-CIP-NA-ETP-TS	1	0	0
AUG-TET-CTX-CAZ-AP-ETP-TS	1	0	0
AUG-TET-ATH-CRO-CPM-CTX-CAZ-NA-ETP-TS	1	0	0
AUG-ATH-CRO-CPM-CTX-CAZ-CIP-NA-ETP-TS	1	0	0
AUG-TET-ATH-CRO-CPM-CTX-CAZ-CIP-NA-AP-TS	1	0	0

CTX = cefotaxime, CAZ = ceptazidime, CPM = cefepime, CRO = ceftriaxone, ATH = azithromycin, AP = ampicillin, AUG = amoxicillin clavulanic acid, ETP = ertapenem, TS = trimethoprim/sulfamethoxazole, NA = nalidixic acid, CIP = ciprofloxacin

Table 3. Distribution of antibiotic resistance genes across the 3 sites. Total DNA was extracted from 21 water samples (7 from each site) and used for PCR analysis. Water samples from Week 1 were not done. For each water sample black boxes indicate presence of gene; white/clear boxes indicate gene not detected.

Drug class	ARG	Upstream							Effluent (WWTP)							Downstream							Total (*N* = 21)
		Wk2	Wk3	Wk4	Wk5	Wk6	Wk7	Wk8	Wk2	Wk3	Wk4	Wk5	Wk6	Wk7	Wk8	Wk2	Wk3	Wk4	Wk5	Wk6	Wk7	Wk8	*N* (%)
Beta-lactams	*SHV*																						0 (0)
	CTX-M	■					■	■	■						■	■		■	■		■	■	10 (47.6)
	KPC																						0 (0)
	NDM									■	■			■									3 (14.2)
Quinolones	*QnrB*						■	■	■		■	■	■	■	■	■	■		■	■	■	■	14 (66.7)
	QnrA						■		■	■	■	■		■	■	■	■	■	■	■	■	■	14 (66.7)
Macrolides	*ErmB*	■								■	■	■	■	■		■	■	■	■		■		11 (52.4)
	ErmA						■			■	■	■	■	■			■	■	■	■	■		11 (52.4)
Sulfamethoxazole	*Sul1*		■		■				■					■	■								5 (23.8)
	Sul2		■	■	■	■	■			■	■	■			■		■	■	■	■	■	■	17 (81)
Trimethoprim	*Dfr14*																						0 (0)

DISCUSSION

Prevalence of antibiotic-resistant *E. coli* across the three sampling sites

Of the 86 isolates that were tested for antibiotic resistance, all were resistant to at least one of the 12 antibiotics tested. The isolates showed elevated resistance (>72%) to AUG, AP, NA, TET, TS, and CIP (Fig. 2). The findings are similar to those of previous studies from around the world, including South Africa (Mbanga et al., 2021), Portugal (Bessa et al., 2014), and Vietnam (Lien et al., 2017). The study by Mbanga et al. (2021) investigated the antibiotic resistance of *E. coli* and *Enterococcus* isolates from a WWTP and its associated river upstream and downstream of the WWTP. A total of 580 *E. coli* isolates were assayed in the study and the highest resistance was observed against AP (63.4%), TS (57.2%), and AUG (53.1%). These percentage resistances were lower than those observed in this study for AP (75.6%), TS (80%), and AUG (72%), probably due to the differences in isolate numbers used and the source of the isolates. In this study, the highest susceptibility of *E. coli* isolates was towards ETP (29%) (Fig. 2). Carbapenems are reserved for treating infections where quinolones and cephalosporins are not effective. The findings in this study are consistent with other studies that have reported higher susceptibility to carbapenems. Lien et al. (2017) reported very low resistance to carbapenems, with only one isolate (out of 265 *E. coli*) from the rural hospital effluent found to be resistant to imipenem. Similarly, a study from Poland (Kotlarska et al., 2015) on 774 *E. coli* isolates from raw and treated effluents of 2 WWTPs and their receiving waters revealed that all isolates were susceptible to both meropenem and imipenem. The results from this study revealed an increased rate of resistance to CTX (40.6%), NA (78.9%) and TET (79%) (Fig. 2). In contrast, a study by Adefisoye and Okoh (2016) on the prevalence of antibiotic-resistant pathogenic *Escherichia coli* from treated wastewater effluents in the Eastern Cape revealed a lower rate of CTX prevalence (4.5%) and reduced resistance to NA (31.4%), and TET (60.1%). However, the South African study only focused on samples from the final effluent, which might explain the observed differences. Another finding from this study was moderate resistance of *E. coli* to cefepime (37.2%), a fourth-generation cephalosporin. Across all three sampling sites, the resistance to cefepime ranged from 29% (upstream site) to 41% (downstream site) (Table 1). These findings are consistent with Tissera et al. (2017) who carried out a study on the isolation of ESBL producing bacteria from urban surface waters in Malaysia and found an increasing emergence of fourth-generation cephalosporins. The emergence of such species in the environment presents a public health risk, especially where communities are known to use the water sources for domestic and recreational purposes, as in the case of Mukuvisi River, especially downstream of the investigated WWTP. The presence of *E. coli* resistant to cephalosporins, quinolones, and carbapenems in the wastewater and associated river water observed in the current study is a cause for concern.

The high level of resistance to the fluoroquinolones nalidixic acid (78.9%) and ciprofloxacin (80.2%) reported in our study is also of concern. The sharp increase in fluoroquinolone-resistant isolates from the upstream site to the effluent and downstream sites needs further investigation.

Fluoroquinolones are considered an effective treatment option for urinary tract infections caused by *E. coli*. The widespread use of quinolones as powerful broad-spectrum antibiotics to treat infections in the respiratory, urinary, and digestive systems leads to their detection in WWTPs regularly around the world. Macrolides and quinolones are also among the antibiotics most consumed worldwide (Van Boeckel et al., 2014).

For most antibiotics that were tested, the results showed a higher percentage of antibiotic-resistant isolates in the final effluent and downstream samples when compared to the upstream site (Table 1). This may imply that treated effluents affect the relative abundance of antibiotic-resistant bacteria in associated surface waters, especially in cases where a final disinfection step is not included in the wastewater treatment. This is supported by the findings of Szczepanowski et al. (2009), who reported that although the wastewater treatment process reduces microbial load by over 99%, antibiotic-resistant bacteria still find their way into the environment, specifically the associated surface waters. The increase in antibiotic-resistant isolates agrees with the findings of Koczura et al. (2014) and Osińska et al. (2017), in which a higher frequency of MDR isolates was reported in the influent and downstream samples than in the upstream samples. This could be explained by the existence of informal settlements at the downstream site in our study, pointing to the possible introduction of ARB from anthropogenic activities at this site.

A total of 81 (94.2%) *E. coli* isolates were MDR, with a total of 48 MDR patterns. The MDR patterns showed that there is potentially a large diversity of resistance determinants haboured by environmental *E. coli*. Some of the observed MDR patterns are shown in Table 2. None of the MDR patterns were common to all 3 sampled sites, with only one pattern being common to the upstream and downstream sites i.e AUG-TET-ATH-CRO-CPM-CTX-CAZ-CIP-NA-AP-TS (Table 2). Three patterns were common to isolates from the effluent and downstream sites. This points to the mobility of isolates and their clones down the river-WWTP continuum, or the constant transfer of mobile genetic elements that confer resistance to the same antibiotics. However, most of the *E. coli* isolates had unique MDR patterns suggesting that the sampled sites could have distinct microenvironments.

Detection of ARGs

Eight out of the eleven antibiotic resistance genes analysed were detected in the wastewater and associated river water samples (Table 3). The results showed that sulfonamide resistance was common across all 3 sampling sites. Sulfonamide resistance in Gram-negative bacteria can probably be attributed to *Sul1* and *Sul2* genes, which are carried by plasmids. In the present study, the *sul2* gene was the common one, as it was observed in 17 (81%) of the samples. This is in agreement with the findings of Stange et al. (2019), who investigated the distribution of clinically relevant antibiotic-resistance genes in Lake Tai, China, and concluded that sulfonamide-resistance genes were the most common ARGs in the environment. Another study by Yan et al. (2018) investigated the occurrence of tetracyclines, sulfonamides, and quinolones and their corresponding resistance genes in the Three Gorges Reservoir in China. Their results demonstrated that sulfonamide-resistance genes were the most ubiquitous ARGs in the environment. Lye et al. (2019) carried out a study to investigate anthropogenic impacts on sulfonamide residues and sulfonamide-resistant bacteria and genes in the Larut and Sangga Besar Rivers, and reported that the *sul2* gene had the highest abundance.

The *qnrA* and *qnrB* genes were also detected in this study from all sampled sites (Table 3). They are known to confer low-level resistance to fluoroquinolones in Enterobacteriaceae and they may be found on the same resistance plasmids as ESBLs (Salah et al., 2019). Research by Colomer-Lluch et al. (2014) revealed the *qnrA* gene as the most prevalent quinolone resistance gene detected in urban wastewater. The presence of *qnrA* and *qnrB* genes in the assayed environments agreed with the high phenotypic resistance to ciprofloxacin and nalidixic acid observed in this study.

ERMB genes detected in this study encode for ribosomal methylase, which is responsible for macrolide resistance (Enne et al., 2001). A study by Pärnänen et al. (2019) investigated the prevalence of ARGs

in 12 WWTPs in European countries and concluded that *emrA* was the most common macrolide-resistant gene detected in WWTPs. This is similar to the results obtained in this study. The incidence of the *ermA and ermB* genes was 52.4% for both genes. This is in agreement with the research of Wang et al. (2020), who investigated the occurrence and fate of antibiotics, antibiotic-resistance genes, and antibiotic-resistant bacteria in municipal wastewater treatment plants. The gene for macrolide resistance, *emrB*, was found to be prevalent in the wastewater samples in their study.

Genes that code for β-lactam resistance were the least detected in this current study, with only CTX-M being detected in only 10 (47.6%) of the samples (Table 3). Although the β-lactam class, involving penicillins and cephalosporins which are the top two most-consumed antibiotics worldwide (Van Boeckel et al., 2014), these compounds are not frequently detected in WWTPs because members of β-lactam antibiotics readily hydrolyze owing to unstable β-lactam rings. The presence of antibiotics in WWTPs contributes to the development of antimicrobial resistance. However, research has shown that the ARGs commonly detected in WWTPs include the genes resistant to β-lactam such as *blaCTX-M*, *blaTEM*, and *blaSHV*.

NDM-1 is an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics including antibiotics of the carbapenems which are a mainstay for the treatment of antibiotic-resistant bacterial infections (Van Boeckel et al., 2014). It encodes β-lactamase enzymes called carbapenemases. The most common bacteria that make these enzymes are Gram-negative *K. pneumoniae* and *E. coli*, but this can be spread from one strain of bacteria to another through horizontal gene transfer (Van Boeckel et al., 2014). However, it's unclear whether and how much of the existing ARGs are carried intercellularly (iARGs) by living, metabolically active bacteria. To go beyond qualitative and quantitative ARG analyses and gain a more comprehensive understanding of ARG dynamics, it is vital to determine which of the discovered ARGs are expressed.

CTX-M genes were detected in lower frequencies across the sites. This is in contrast with a study done in South Africa (Adegoke et al., 2020), where $bla_{CTX\text{-}M}$ was found in 52.6% ($n = 38$) of the samples. NDM genes have also been reported in the current study but at very low frequencies across the sampling sites. A study done in China also confirmed the occurrence of $bla_{NDM\text{-}5}$ in wastewater (Zhang et al., 2016).

CONCLUSION

The results of our study indicate that a high proportion of MDR *E. coli* were present in the investigated WWTP and its receiving waters. The *E. coli* had a great diversity of antibiotic-resistance profiles, implying that the WWTP and its receiving river are important reservoirs of ARGs and antibiotic-resistant bacteria, which are potential human and animal pathogens. Antibiotic-resistance genes belonging to several drug classes were detected at the sampled sites directly from wastewater. The presence of antibiotic-resistant *E. coli* and ARGs in environmental waters presents a significant health risk to the people and animals that use these water sources and could be potential reservoirs for the dissemination of antibiotic resistance.

AUTHOR CONTRIBUTIONS

Co-conceptualized the study: JM, HT. Performed the experiments: HT, JM. Analysed the data: HT, JM. Wrote the paper: JM, HT. Supervision: JM. Undertook critical revision of the manuscript: All.

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COMPETING INTERESTS

The authors declare that they have no conflicting interests regarding the publication of this paper.

ETHICAL APPROVAL

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APPENDIX

Table A1. Antibiotic resistance gene primers used in this study

Primer	Primer sequence	Annealing temperature	PCR product size	Reference
QNRB-F	5′-GGMATHGAAATTCGCCACTG-3′	57	469	Cattoir et al., 2007
QNRB-R	5′-TTTGCYGYYCGCCAGTCGAA-3′			
DFR14	5′-TGAGAACCTTGAAAGTATCATTG-3′ 5′-ACCCTTTTTCCAAATTTGATAG-3′	55	483	Rahmani et al., 2013
CTX-M	5′-ATGTGCAGYACCAGTAARGTKATGGC-3′ 5′TGGGTRAARTARGTSACCAGAAYCAGCGG-3′	60	593	Hasman et al., 2006
KPC	5′-CGTCTAGTTCTGCTGTCTTG-3′ 5′-CTTGTCATCCTTGTTAGGCG-3′	55	636	Poirel et al., 2011
Sul1	5′-TGAGATCAGACGTATTGCGC-3′ 5′-TTGAAGGTTCGACAGCACGT-3′	57	417	Rahmani et al., 2013
QNRA	5′-GGATGCCAGTTTCGAGGA-3′ 5′-TGCCAGGCACAGATCTTG-3′	57	516	Cavaco et al., 2008
Sul2	5′-GCGCTCAAGGCAGATGGCATT-3′ 5′-GCGTTTGATACCGGC ACCCGT-3′	55	249	Frank et al., ND
ERM B	5′-GGAACATCTGTGGTATGGCG-3′ 5′-CATTTAACGACGAAACTGGC-3′	60	738	Jensen et al., 1999
ERM A	5′-AAGCGGTAAAACCCCTCTGAG-3′ 5′-TCAAAGCCTGTCGGAATTGG-3′	60	139	
SHV	5′-TTATCTCCCTGTTAGCCACC-3′ 5′-GATTTGCTGATTTCGCTCGG-3′	60	713	Arlet et al., 1997
NDM	5′-GGTTTGGCGATCTGGTTTTC-3′ 5′-CGGAATGGCTCATCACGATC-3′	55	600	Poirel et al., 2011

Sul1 *and* Sul2 = *sulfonamide resistant gene, NDM = New Dehli metallo-beta-lactamase, SHV = sulfhydryl reagent variable, INT1 = Class I integron, QNR = quinolone resistance gene, ERM = erythromycin resistance methylase gene, KPC* = Klebsiella pneumoniae *carbapenemase, CTX-M = cefotaxime Munich, DFR = dihydrofolate reductase*