The Abundance of Genes Encoding ESBL, pAmpC and Non-β-Lactam Resistance in Multidrug-Resistant Enterobacteriaceae Recovered From Wastewater Effluents

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The aquatic environments play a critical function in the widespread of antibiotic-resistant bacteria, ultimately impacting human health. We evaluated the abundance of Enterobacteriaceae and the various resistance gene determinants in final effluents of wastewater treatment plants (WWTPs) in the Eastern Cape Province, Republic of South Africa. A total of 44 presumptive Enterobacteriaceae was recovered following standard isolation methods. Upon molecular confirmation through Matrix-Assisted Laser Desorption/Ionization Time of flight (MALDI TOF), a high predominance of *Klebsiella pneumoniae* (24%) was noted. All the confirmed isolates (n = 29) subjected to a panel of eighteen antibiotics categorized into eleven different classes were multidrug-resistant (MDR). They displayed resistance against antibiotics in more than three different classes. Notably, one *E. cloacae* exhibited resistance against all the antibiotics assayed. The multiple antibiotic resistance indices (MARI) ranged from 0.22 to 1.0, indicating an environment with high pressure of antibiotics. Conventional Polymerase Chain Reaction (PCR) analysis showed that 72.4% of the isolates harboured at least one β-lactamase genetic determinant. The most predominant extended-spectrum β-lactamases were $bla_{CTX-M-group}$ (21.5%), bla_{TEM} (20%), and bla_{SHV} (16.9%) while the most predominant plasmid-mediated AmpC-type β-lactamases were bla_{CIT} and bla_{ACC} (25.9%) and bla_{EBC} (22.2%). A total of 86.2% of the Enterobacteriaceae harboured a minimum of one non-β-lactam resistance gene determinant with predominance observed in *catII* (25%), *sulII* (15.8%), and *sulI* (14.5%). These results demonstrate an abundance of multidrug-resistant (MDR) Enterobacteriaceae in WWTPs' effluents. The study confirms the need to optimize current WWTPs' processes to improve the quality of effluents and ultimately reduce the potential risks of using such water when discharged into the environment.

Keywords: wastewater treatment plant, extended—spectrum β—lactamases, plasmid-mediated AmpC beta-lactamase, enterobacteriaceae, multidrug-resistance

INTRODUCTION

The most common zoonotic pathogens are found in the Enterobacteriaceae family. They are common in water environments and can cause infections in both humans and animals. They can cause various illnesses like diarrhoea, septicaemia, enteritis, and urinary tract infections (Tärnberg, 2012). A typical aquatic environment that harbours these Enterobacteriaceae is the wastewater treatment plant (WWTP), which offers beneficial circumstances for the rapid growth of a diverse bacterial community. These WWTPs are recipients of various categories of wastes from hospitals, households, and industries. Hence, they serve as an essential reservoir of microorganisms that reach the environment through the effluents and infect humans and animals if not adequately treated. It has been reported that WWTPs, even when functioning at optimum, do not eradicate antibiotic-resistant bacteria (ARB) and their genes. Municipal WWTPs have been principally designed to remove nutrients and reduce bacterial load to certain acceptable limits (Okoh et al., 2010; Yu et al., 2018; Aristizábal-Hoyos et al., 2019). When antibiotics are used, the body does not fully metabolize them and are discharged as wastes into the sewer system and finally emptied into the WWTPs, where they are only partially removed (Lamprecht et al., 2014; Adefisoye and Okoh 2016). As a result, antibiotics in wastewater can exert selective pressure on ARB, encouraging their spread in widely varied environments.

Antibiotics of various categories are used to treat infections mediated by the Enterobacteriaceae family. Due to the usage and overuse of antibiotics, ARB proliferation in clinical and veterinary settings arose because of selective pressure generated. The ARB in Enterobacteriaceae is currently considered a significant public health issue with critical priority by the World Health Organization (WHO) (WHO, 2014). There are varying defence mechanisms that bacterial species employ to evade the effects of antibiotics administered against them (Jacoby, 2009). In clinical practice, β-lactam antibiotics are among the most important therapeutic options against infections mediated by members of Enterobacteriaceae (Li et al., 2019). Over time, these microorganisms developed the ability to hydrolyze the chemical backbone in the β-lactams through enzymes, β-lactamases (Rawat and Nair, 2010; Cantón et al., 2012).

β-lactamases are greatly diversified due to their ability to continuously mutate. In this category, the extended-spectrum β-lactamases (ESBLs) have been reported as a great concern in clinical practice (Jacoby, 2009; Bush and Jacoby, 2010; Cantón et al., 2012). The ESBLs are the class A β-lactamases that can hydrolyze penicillins, penicillin inhibitors, monobactams, and third and fourth-generation cephalosporins and are suppressed by clavulanic acid (Paterson and Bonomo, 2005; Bush and Jacoby, 2010). There has been a significant evolution of these ESBLs with high dissemination potential, especially in clinical settings. The genotype first reported was the TEM (Datta and Kontomichalou, 1965; Bush and Jacoby, 2010), followed by the SHV (Brunton et al., 1986; Bush and Jacoby, 2010). In 1988, the first CTX-M group, the CTX-M-1, was detected (Bauernfeind et al., 1990; Bush and Jacoby, 2010). The CTX-M family, which includes CTX-M-25, CTX-M-9, CTX-M-8, CTX-M-1, and CTX-M-2, is the most predominant ESBL genotype. Several other β-lactamases of clinical importance have been identified. These include the minor ESBLs, which are the PER, GES, and VEB. Other important β-lactamases, such as the carbapenemases, include IMP, KPC, VIM, OXA-1-like, and OXA-48-like (Paterson and Bonomo, 2005; Dallenne et al., 2010). Moreover, the pAmpCs are the class C β-lactamases and include six families; CIT, ACC, EBC, DHA, MOX, and FOX. However, they are closely related to ESBLs but display some differences. In contrast to the ESBLs, the pAmpCs are resistant to a broader range of β-lactams and cannot be inhibited by clavulanic acid (Jacoby, 2009). The β-lactams hydrolyzed by ESBLs, carbapenemases, and pAmpCs are critically important therapeutic agents for humans (WHO, 2017).

The resistance of β-lactamases producers against multiple antibiotic classes makes them of great concern in clinical settings (Rawat and Nair, 2010; Paterson, 2000). It makes the treatment of patients infected by them difficult and often almost impossible. The resistance genes for the ESBLs and pAmpCs are regularly encoded on plasmids with large sizes. These plasmids concurrently encode other resistance genes which belong to other antimicrobials classes. The non-β-lactams include tetracycline, trimethoprim, sulphonamide, chloramphenicol, fluoroquinolone, and aminoglycoside (Kotlarska et al., 2015). The spread of bacterial pathogens concurrently harbouring these resistance genetic determinants is of particular concern as it further limits clinical treatment options. Despite resistance exhibited by bacteria against antibiotics, they still serve as a gold-therapy choice for treating infections caused by bacteria. Therefore, due diligence must be taken to decrease the global dissemination of antibiotic resistance. Therefore, this research aimed to assess the total quantification of the Enterobacteriaceae community present in the WWTPs' effluents. In addition, to determine the abundance of the ESBLs, pAmpCs, and other non-β-lactam encoding genes in the Enterobacteriaceae isolated at the point of discharge into communal surface water.

MATERIALS AND METHODS

Description of Study Sites

The study was carried out in two District Municipalities (DMs) within the Eastern Cape Province (ECP) Republic of South Africa (RSA), the Amathole and Chris Hani DMs (ADM and CHDM, respectively). In ADM, the WWTP is in Alice, a semi-urban settlement in the Raymond Mhlaba Local Municipality with geographical coordinates 32°50′36″ S and 26°55′00″ E. The WWTP, which uses the activated sludge technology, receives light industrial wastewater, run-off water, and residential sewage. The WWTP is comprised of a screen channel, aerobic and anaerobic chambers, and a clarifier. The effluent is chlorinated before it is released into the Tyhume River. The plant's capacity was designed for 2000 m^3/day but was operating at about 1,100 m^3/day, which is 55% of its designed capacity during the period of sample collection.
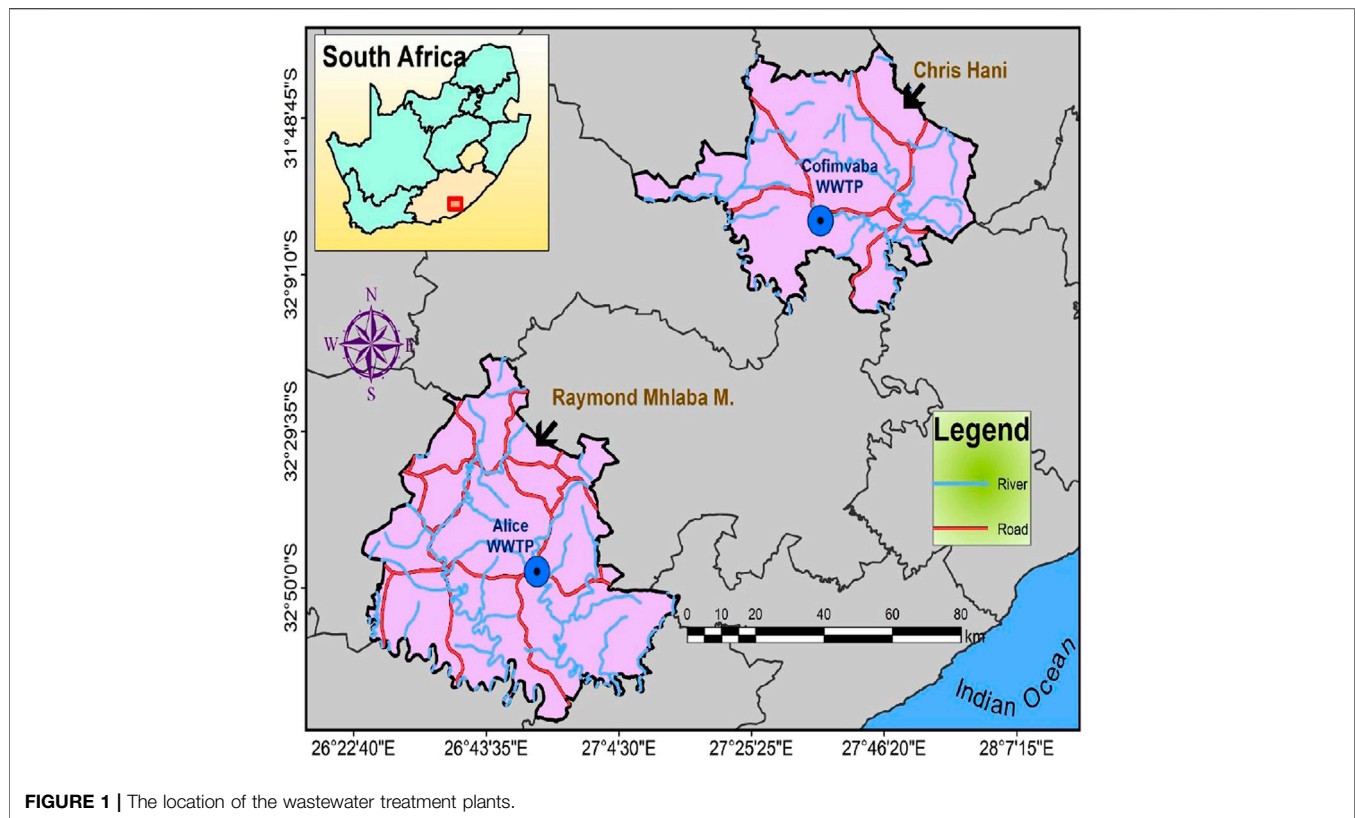
FIGURE 1 | The location of the wastewater treatment plants.

The WWTP for the second DM, the CHDM, is located in Cofimvaba, another semi-urban settlement located at 32°03′23″ S and 27°60′08″ E. Domestic sewage, run-off waters, and some light industrial wastewater are all treated at this facility. It also uses an activated sludge technology treatment facility which includes systems similar to those used at the Alice WWTP described above. The final chlorinated effluent is drained down the Cofimvaba River, which finally empties into the Great Kei River. A map showing the location of the sampling sites is shown in **Figure 1**.

Sample Collection

A total of six wastewater samples were obtained from WWTPs in October and November 2017. For de-chlorination, the treated final effluents exiting the end-of-pipe were collected into sterile 1-L polypropylene containers into which 1.7 ml of 1 per cent sodium thiosulfate solution has been dispensed (Standard Methods, 2005). Within 6 h of sample collection, they were transported to the lab on ice for microbiological testing. Samples were held at 4°C until preliminary steps were executed.

Quantification and Isolation of Presumptive Enterobacteriaceae

A ten-fold serial dilution was carried out using sterile distilled water. Every 100 ml of adequately diluted samples was filtered using a vacuum filtration pump. The membrane filters were aseptically transferred to the Violet Red Bile Glucose (VRBG) agar (Merck, Modderfontein, RSA) and incubated at 37°C for 18 h. Purple-red colonies were then counted as Enterobacteriaceae, with the results designated as colony-forming units per 100 ml (CFU/100 ml). For the isolation of presumptive *Enterobacter* spp., *Citrobacter* spp., *E. coli*, and *Klebsiella* spp., the different colonies on VRBG were then picked unto MacConkey agar and Eosin Methylene Blue agar (Conda, Pronadisa, RSA). The purified presumptive isolates were stored at −80°C in 25% glycerol stock for further laboratory analysis.

Molecular Confirmation of Presumptive Enterobacteriaceae

The molecular identities of the purified presumptive isolates were done using Matrix-Assisted Laser Desorption Ionization (MALDI-TOF) coupled with Mass Spectrometry (MS) using the Biotyper 3.0 protocol (Bruker Daltonics, Germany) as previously described (Standing et al., 2013). All isolates were tested in duplicates. For quality control, Bacterial Test Standard (BTS) (8255343) was included in every plate. The manufacturer's guideline was used in the interpretation of the results obtained. Score values lower than 1.70 were excluded, as the identification is considered unreliable (Standing et al., 2013).

Antimicrobial Susceptibility Profile Using Disk Diffusion Assay

The disk diffusion protocol, following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018), was used to determine the antimicrobial susceptibility profile of all the confirmed bacterial isolates after identification using MALDI-TOF MS. The list of antibiotics evaluated were: cefuroxime (CXM/30 μg) and cefotaxime (CTX/30 μg) belonging to the cephems, chloramphenicol (C/30 μg) belonging to the phenicols, amoxicillin/clavulanic acid (AUG/30 μg) and ampicillin (AP/10 μg) belonging to the β-lactams, ciprofloxacin (CIP/5 μg) and norfloxacin (NOR/30 μg) belonging to the fluoroquinolones, doxycycline (DXT/30 μg) and tetracycline (T/30 μg) belonging to the tetracyclines, amikacin (AK/30 μg) and gentamicin (GM/10 μg) belonging to aminoglycosides, colistin (CO/25 μg) and polymyxin B (PB/300 units) belonging to the polymyxins, nitrofurantoin (NI/300 μg) belonging to the nitrofurans, nalidixic acid (NA/30 μg) belonging to the quinolones, meropenem (MEM/10 μg) and imipenem (IMI/10 μg) belonging to the carbapenems and lastly, trimethoprim-sulfamethoxazole (TS/25 μg) belonging to the sulphonamides (Mast Diagnostics, United Kingdom).

Assessment of Multiple Antibiotic Resistance Phenotypes and Multiple Antibiotic Resistance Indices

By analyzing the antibiotic susceptibility of these isolates, an isolate was classified as multidrug-resistant (MDR) when it exhibited resistance against at least one antimicrobial agent in at least three different antibiotic groups, as previously described (Magiorakos et al., 2012). The MARPs were consequently assessed as earlier described (Ateba and Bezuidenhout, 2008). The MARI of the bacterial species was derived as defined following the mathematical calculation (Krumperman, 1983):

$$MAR\ index = c/d$$

Here "c" refers to the sum of antibiotics the bacterial species displayed phenotypic resistance against, while "d" refers to the total number of antibiotics assessed. A MARI score of more than 0.2 indicates that antibiotics are intensively used in that area and implies an environment with a high risk of antimicrobial resistance proliferation (Krumperman, 1983; Osundiya et al., 2013).

DNA Extraction

Genomic DNA used for Polymerase Chain Reaction (PCR) experiment was extracted using the boiling method as reported previously (Maugeri et al., 2004).

Molecular Detection of Antimicrobial Resistance Gene Determinants

All of the isolates were subjected to molecular characterization of important β-lactamases in the Enterobacteriaceae family using multiplex, and duplex PCR protocols with the aid of a Biorad

Thermal Cycler (United States), under conditions defined by Dallenne and colleagues (Dallenne et al., 2010). The various resistance genes encoding the different β-lactamases variants assayed were; bla_{TEM} (comprising TEM-2 and TEM-1), $bla_{CTX-M-9}$ (comprising CTX-M-14 and CTX-M-9), bla_{SHV} (SHV-1), $bla_{CTX-M-1}$ (comprising CTX-M-15, CTX-M-1, and CTX-M-3), $bla_{CTX-M-2}$ (comprising CTX-M-2), bla_{PER} (comprising PER-3 and PER-1), bla_{VEB} (containing VEB-1 through VEB-6), bla_{GES} (including GES-11 and GES-1 through GES-9), bla_{IMP} (excluding IMP-25, IMP-22, IMP-18, IMP-16, and IMP-9), $bla_{OXA-1-like}$ (comprising OXA-30, OXA-4, and OXA-1), bla_{VIM} (including VIM-2 and VIM-1), bla_{KPC} (containing KPC-1 to KPC-5), and $bla_{OXA-48-like}$. In the same vein, pAmpC-type β-lactamase variants mediated by plasmids, namely CIT, ACC, EBC, DHA, MOX, and FOX, were also assayed. They were organized into the following various categories. The bla_{CIT} (BIL-1, CMY-12 to CMY-18, CMY-2 to CMY-7, LAT-1 to LAT-3, and CMY-21 to CMY-23), bla_{ACC} (including ACC-2 and ACC-1), bla_{EBC} (MIR-1 and ACT-1), bla_{DHA} (DHA-2 and DHA-1), bla_{MOX} (CMY-19, MOX-1, CMY-1, MOX-2, and CMY-8 to CMY-11), and bla_{FOX} (including FOX-1 to FOX-5). Genes encoding non-β-lactam resistance were investigated for all isolates as described previously (Titilawo et al., 2015) for tetracyclines (tetB, tetD, tetC, tetM, tetK, and tetA), sulphonamides (sulII and sulI), and phenicols (catII and catI). All primers were produced by Inqaba Biotechnological Industries (Pretoria, RSA). A volume of 5 μL of the amplified DNA was resolved in a 1.5% (w/v) agarose gel (Separations, RSA) stained with 5 μL ethidium bromide. A molecular marker of 100 base pairs (Thermo Scientific, United States) was used. The gel electrophoresis was run in 0.5X Tris-borate EDTA buffer for 1 h at 100 V and visualized in a UV transillumination (ALLIANCE 4.7, UVIec, Merton, London, United Kingdom).
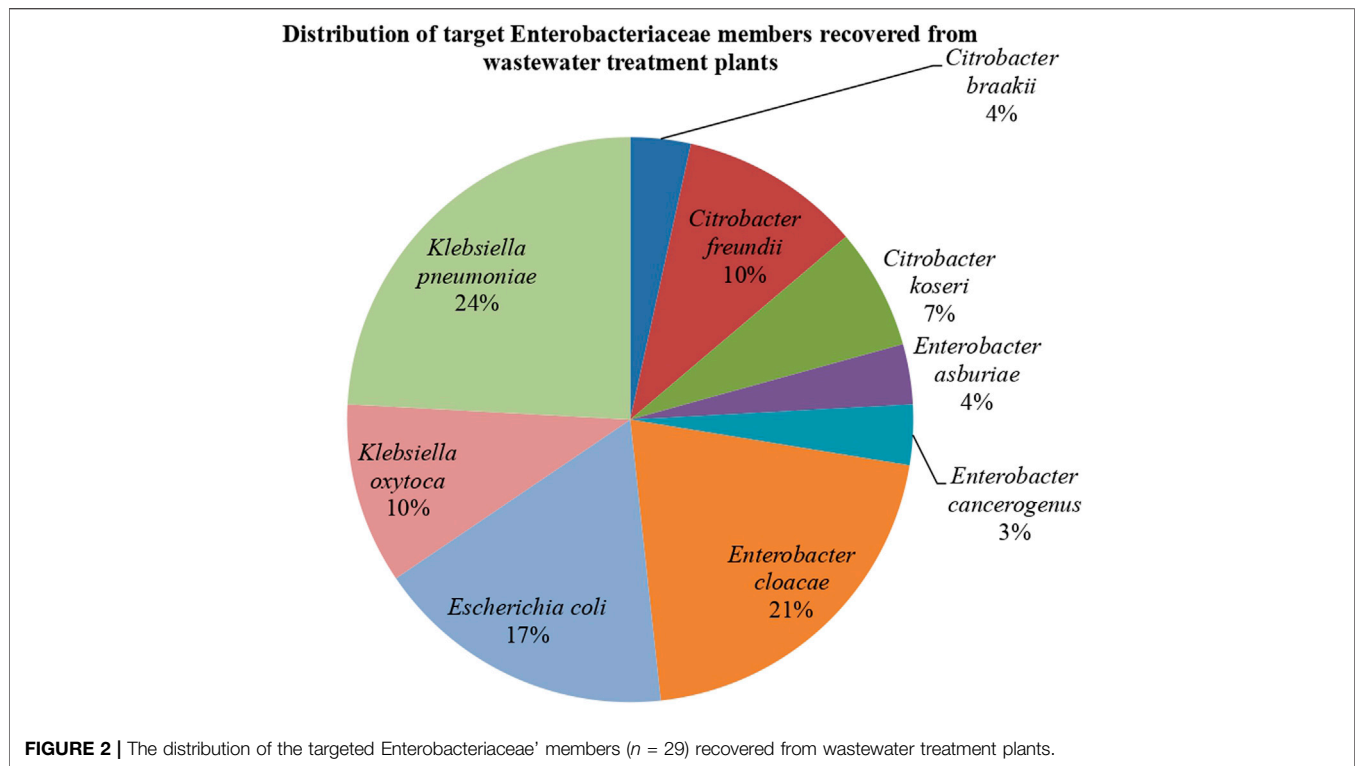
Data Analysis

The data acquired were analyzed utilizing the descriptive statistical program available in Microsoft Excel 2010.

RESULTS AND DISCUSSION

Total Quantification and Identification of Enterobacteriaceae in the Wastewater Treatment Plants

Water quality is a significant concern for many communities that rely on freshwater sources for consumption and recreation purposes. Concerns being raised regarding their pollution by pathogens (Sibanda et al., 2013) and on the requirement by law (Department of Water Affairs (DWAF), 2012), which states that treated effluents must be discharged into public streams or seas where the water was initially obtained. In this study, the average counts of the total Enterobacteriaceae bacteria were 3.37×10^4 CFU/100 ml and 5.75×10^5 CFU/100 ml in ADM and CHDM effluents, respectively. Since these effluents are being discharged into the surrounding surface water, the bacterial

FIGURE 2 | The distribution of the targeted Enterobacteriaceae' members (*n* = 29) recovered from wastewater treatment plants.

counts observed was compared with the compliance concentrations of the South African and WHO tolerance limits for effluents intended for discharge by municipal WWTPs into the receiving surface water. Properly treated effluent should not have a total bacterial count greater than 10^4 CFU/100 ml. The results obtained in this study is higher than the threshold of the bacterial count for municipal WWTP final effluents. These values are an indication that the effluents that are discharged into the surrounding surface water are of poor quality as enteric microorganisms are not adequately eliminated or reduced to acceptable limits. The results obtained are an indication that bacterial species are widely distributed in the WWTPs effluents. The quality of an effluent discharge is particularly an important criterium in assessing the efficiency of a WWTP. The surface water which receives these municipal WWTP effluents are being abstracted for irrigation, domestic, and religious activities (Fadare et al., 2020), thereby being a public health concern as they are unsuitable for domestic uses.

In total, 44 presumptive bacterial isolates were recovered from the different selective agar. These bacterial species belonged to four different families, which are Moraxellaceae (1), Morganellaceae (2), Staphylococcaceae (1), and Enterobacteriaceae (35). The remaining five bacterial species had a matching value lower than 1.7, which is the minimum score value for the reliability of the identity of an isolate using MALDI-TOF and were consequently excluded from this study. From the Enterobacteriaceae family, six genera were identified, which include Serratia (2), Proteus (4), Klebsiella (10), Escherichia (5), Enterobacter (8), and Citrobacter (6) as shown in **Supplementary Table S1**. The present study focuses on the last

four genera of the Enterobacteriaceae family (*n* = 29). Among these, the most frequently detected bacterial species was *K. pneumoniae* (7/29; 24%), followed by *E. cloacae* (6/29; 21%) and *E. coli* (5/29; 17%). The other bacterial species identified were *K. oxytoca* and *C. freundii* (3/29; 10% each), *C. koseri* (2/29; 7%), and *C. braakii*, *E. asburiae*, and *E. cancerogenus* (1/29; 3% each), as shown in **Figure 2**. The detection and subsequent isolation of these Enterobacteriaceae indicate that the bacterial species were not eliminated during the various treatment stages in the WWTP. Enterobacteriaceae are members of the Proteobacteria phylum, which is distinguished by their capability of transferring DNA to bacteria from other classes. They can also promote genetic exchange between environmental and commensal bacteria in water and other environments (Ojer-Usoz et al., 2014). Different Enterobacteriaceae genera and species were found in this study, close to those found in the WWTPs of other nations, including Portugal, Colombia, and Spain (Ojer-Usoz et al., 2017; Aristizábal-Hoyos et al., 2019; Teixeira et al., 2020). These studies reported that members of the Enterobacteriaceae could survive the biological treatment processes employed in the management of municipal WWTPs.

Antibiogram Profiling of Targeted Enterobacteriaceae From Wastewater Treatment Plants

The antibiotic susceptibility profiles of the confirmed bacterial species recovered from final effluents of WWTPs are shown in **Figure 3**. Concerning the individual genera, all the *Klebsiella* spp. which were isolated exhibited resistance against three antibiotics.

FIGURE 3 | The Antimicrobial susceptibility profiling of members of the Enterobacteriaceae family recovered from municipal wastewater treatment plants indicating the extended-spectrum β-lactamase and carbapenemases, plasmid-mediated AmpC β-lactamase, and non-β-lactam resistance determinants. The colour code of the antimicrobial susceptibility profiles shows the phenotypes of the isolates to specific antibiotics in 11 different classes. The detection of the resistance gene determinants is indicated as present or absent.

These are doxycycline, ampicillin, and cefotaxime which are from different antibiotic classes.

In *E. coli*, all the isolates exhibited resistance against ampicillin, cefotaxime, amoxicillin/clavulanic acid, and nalidixic acid. However, none of the *E. coli* strains displayed resistance against imipenem or amikacin. All the species of Enterobacter were resistant to four different antibiotic classes. These classes

were the β-lactams, polymyxins, tetracyclines, and nitrofurantoin. The least phenotypic resistance observed was against meropenem and amikacin, with only one *E. cloacae* strain (Isolate No. 26) exhibiting resistance against these antibiotics. Interestingly, 1 *E. cloacae* (Isolate No. 22) showed resistance against eighteen antibiotics assayed (**Figure 3**). All the *Citrobacter* spp. were resistant against amoxicillin-clavulanic

TABLE 1 | The patterns of the MARPs and MARI of Enterobacteriaceae from municipal WWTPs' effluents.

S/N	MAR phenotypes	No. of the antibiotics	No. of the isolates	MARI
	Klebsiella species (n = 10)			
1	DXT, T, TS, NA, C, NI, NOR, CIP, CXM, CTX, MEM, IMI, AP, AUG, AK, GM	16	1	0.89
2	DXT, TS, NA, CO, PB, C, NI, NOR, CIP, CXM, CTX, AP, AUG, AK, GM	15	1	0.83
3	DXT, T, TS, NA, PB, C, NI, NOR, CIP, CXM, CTX, AP, AUG, AK, GM	15	1	0.83
4	DXT, T, TS, NA, CO, PB, C, NI, NOR, CIP, CXM, CTX, AP, AUG, GM	15	3	0.83
5	DXT, TS, NA, CO, PB, C, NOR, CIP, CXM, CTX, AP, AUG, GM	13	1	0.72
6	DXT, T, NA, CO, PB, C, NI, CIP, CTX, MEM, AP, AUG	12	1	0.67
7	DXT, T, TS, NA, CO, C, NI, CTX, AP, AUG	10	1	0.56
8	DXT, T, CO, PB, NI, CTX, AP	7	1	0.39
	E. coli (n = 5)			
1	DXT, T, TS, NA, C, NI, NOR, CIP, CXM, CTX, MEM, AP, AUG, GM	14	1	0.78
2	DXT, T, TS, NA, CO, PB, C, NI, NOR, CIP, CXM, CTX, AP, AUG, GM	15	3	0.83
3	NA, CTX, AP, AUG	4	1	0.22
	Citrobacter species (n = 6)			
1	DXT, T, TS, NA, C, NI, CXM, CTX, MEM, AP, AUG, AK, GM	13	1	0.72
2	DXT, T, TS, NA, C, CXM, CTX, MEM, AP, AUG, AK, GM	12	1	0.67
3	DXT, T, TS, NA, CO, NI, PB, AP, AUG, GM	10	1	0.56
4	DXT, T, TS, C, NI, CTX, AP, AUG	8	1	0.44
5	DXT, T, TS, NA, CO, PB, C, NI, CXM, AP, AUG	11	1	0.61
6	TS, NI, AP, AUG	4	1	0.22
	Enterobacter species (n = 8)			
1	DXT, T, TS, NA, CO, PB, C, NI, NOR, CIP, CXM, CTX, MEM, IMI, AP, AUG, AK, GM	18	1	1
2	DXT, T, TS, NA, CO, PB, C, NI, NOR, CIP, CXM, CTX, AP, AUG, GM	15	2	0.83
3	DXT, TS, NA, CO, PB, C, NI, CXM, CTX, AP, AUG, GM	12	1	0.67
4	DXT, T, TS, NA, CO, PB, C, NI, CTX, AP, AUG, GM	12	1	0.67
5	DXT, T, CO, PB, C, NI, IMI, AP, AUG	9	1	0.5
6	DXT, T, TS, CO, PB, NI, CIP, CXM, CTX, AP, AUG	11	1	0.61
7	DXT, T, NA, CO, PB, C, NI, CXM, CTX, AP, AUG	11	1	0.61

Antibiotics code: TS–trimethoprim/Sulfamethoxazole, GM–gentamicin, CTX–cefotaxime, AK–amikacin, AM–ampicillin, CXM–cefuroxime, AUG–amoxicillin/clavulanic acid, MEM–meropenem, IMI–imipenem, NI–nitrofurantoin, CO–colistin, NOR–norfloxacin, C–chloramphenicol, DXT–doxycycline, PB–polymyxin B, CIP–ciprofloxacin, and T–tetracycline.

acid, ampicillin, and trimethoprim-sulfamethoxazole but were all susceptible to imipenem, ciprofloxacin, and norfloxacin.

Generally, across all the Enterobacteriaceae members, some resistance was observed against all the antibiotics. The least resistance by the isolates was observed against amikacin and carbapenems. Carbapenems have been considered a drug of last resort. They are generally not prescribed as a front-line antimicrobial, so the low rate detection of resistance against these antimicrobials is unsurprising.

The Multiple Antibiotic Resistance Phenotype and Multiple Antibiotic Resistance Indice of Enterobacteriaceae From Wastewater Treatment Plants Effluents

The various MARP patterns and MARI displayed by the various genera of the Enterobacteriaceae are presented in **Table 1**. All the bacterial species displayed resistance against a minimum of three different classes of antibiotics out of the eleven classes assayed and were thus classified as MDR (Magiorakos et al., 2012). The average MARI obtained for all the isolates was 0.65, and it ranges from 0.22 to 1.0. Almost all the MARP patterns observed occurred singly except a

pattern of resistance against 15 antibiotics observed in three *E. coli* strains. All the isolates have a MARI value greater than the benchmark of 0.2, indicating a higher than normal pressure of antibiotics in the WWTPs. Most of the antibiotics administered in the clinical or veterinary settings are incompletely broken down in the body and are thus excreted into the wastewater systems. Antibiotics in wastewater have been shown to exert selective pressure on antimicrobial-resistant bacterial organisms, promoting their dissemination into other habitats. (Li et al., 2019).

Generally, the results demonstrate that WWTPs are hotspots for shedding enteric pathogens with resistance to multiple classes of antibiotics into the receiving watersheds and, ultimately, into the environment. Many other researchers have reported this in their findings (Okoh et al., 2010; Korzeniewska and Harnisz, 2013; Adefisoye and Okoh, 2016; Igbinosa, 2016; Aristizábal-Hoyos et al., 2019). The results of the MAR index indicate that 41% (12/29) of the isolates recovered from the WWTPs exhibited resistance against a minimum of fifteen of the tested antibiotics. According to previous studies, wastewater, even after treatment, contains a proportion of different resistant bacterial populations (Huang et al., 2012; Ke et al., 2013; Amador et al., 2015; Guyomard-Rabenirina et al., 2017). The conditions in the WWTPs, according to these studies, are conducive to ARB

TABLE 2 | The patterns of the various multiple antibiotic resistance genotypes of Enterobacteriaceae recovered from wastewater treatment plants effluents.

S/N	MAR genotypes	No. of ESBL and carbapenemases	No. of pAmpC genes	No. of non-β-lactam resistance genes	No. of observed pattern
	Klebsiella spp.				
1	bla_{TEM}-bla_{SHV}	2	0	0	1
2	bla_{TEM}-bla_{SHV}-bla_{VIM}-bla_{IMP}-bla_{CIT}-$tetA$-$tetM$	4	1	2	1
3	bla_{TEM}-bla_{SHV}-$bla_{CTX-M-2}$-bla_{VIM}-$catII$-$sulII$-$tetA$-$tetM$	4	0	4	1
4	$catII$-$sulII$	0	0	2	3
5	bla_{TEM}-bla_{KPC}-bla_{GES}-bla_{PER}-bla_{CIT}-bla_{MOX}-$tetB$-$tetD$	4	2	2	1
6	bla_{TEM}-bla_{SHV}-bla_{KPC}-$bla_{CTX-M-2}$-bla_{DHA}-$catII$-$sull$	4	1	2	1
7	bla_{TEM}-bla_{SHV}-$bla_{CTX-M-2}$-bla_{DHA}-$catII$-$sulII$-$sull$	3	1	3	1
	E. coli				
1	bla_{TEM}-bla_{SHV}-$bla_{CTX-M-9}$-$bla_{OXA-1-like}$-bla_{IMP}-bla_{EBC}-bla_{CIT}-$catII$-$tetA$-$tetC$-$tetD$	5	2	4	1
2	$bla_{CTX-M-1}$-$catII$-$sulII$-$sull$-$tetA$-$tetB$	1	0	5	1
3	bla_{TEM}-$bla_{CTX-M-1}$-$bla_{CTX-M-2}$-$bla_{OXA-48-like}$-bla_{IMP}-bla_{ACC}-bla_{EBC}-bla_{MOX}-$catII$-$sulII$-$tetB$-$tetC$-$tetD$-$tetK$	5	3	6	1
4	bla_{TEM}-bla_{SHV}-$bla_{CTX-M-1}$-$bla_{CTX-M-2}$-$bla_{OXA-48-like}$-$bla_{OXA-1-like}$-bla_{IMP}-bla_{ACC}-bla_{EBC}-bla_{DHA}-bla_{MOX}-$catII$-$sulII$-$sull$-$tetB$-$tetC$-$tetD$-$tetK$	7	4	7	1
5	$bla_{CTX-M-2}$-bla_{ACC}-$catII$	1	1	1	1
	Enterobacter spp.				
1	bla_{ACC}-bla_{EBC}-bla_{CIT}-$catII$-$catI$-$tetA$	0	3	3	1
2	bla_{SHV}-$bla_{CTX-M-2}$-$bla_{OXA-1-like}$-bla_{GES}-bla_{IMP}-bla_{ACC}-bla_{EBC}-bla_{CIT}-$catII$-$sulII$-$sull$-$tetA$-$tetB$-$tetC$-$tetM$	5	3	7	1
3	bla_{IMP}-bla_{CIT}-$catII$	1	1	1	1
4	bla_{TEM}-$catII$	1	0	1	1
5	bla_{TEM}-bla_{SHV}-$bla_{CTX-M-2}$-$bla_{OXA-48-like}$-$bla_{OXA-1-like}$-bla_{IMP}-$catII$-$sull$-$tetA$-$tetD$-$tetM$	6	0	5	1
	Citrobacter spp.				
1	bla_{TEM}-bla_{SHV}-$bla_{OXA-48-like}$-$bla_{OXA-1-like}$-bla_{PER}-bla_{IMP}-bla_{ACC}-$sulII$-$sull$-$tetM$	6	1	3	1
2	bla_{TEM}-bla_{SHV}-$bla_{CTX-M-9}$-$bla_{OXA-1-like}$-bla_{EBC}-bla_{CIT}-$catII$-$sulII$-$sull$-$tetA$	4	2	4	1
3	$bla_{CTX-M-2}$-bla_{ACC}-$catII$-$sull$-$tetC$-$tetD$	1	1	4	1
4	$bla_{OXA-1-like}$-$catII$-$tetA$	1	0	2	1
5	bla_{FOX}-$sulII$-$sull$-$tetA$	0	1	3	1

proliferation, which could lead to the transmission of antibiotic-resistant genes to non-resistant bacteria.

Multiple Antibiotic Resistance Genotypes of Bacterial Species Recovered From Wastewater Treatment Plants' Effluents

Findings from the antibiotic-resistant genes (ARGs) investigated in this study revealed that the isolates harboured various resistance genes responsible for the multiple phenotypic resistance displayed by the bacterial species. These resistance genes were broadly classified as the β-lactamases and the non-β-lactam resistance genes.

Out of all the Enterobacteriaceae investigated in the study, 86.2% ($n = 25$) harboured at least one of the various ARGs investigated. As indicated in **Table 2**, the MARG patterns of these microorganisms occurred uniquely. Three *K. pneumoniae* strains (Isolate No 4, 5, and 6) were positive to only two of the resistance genes assayed: *catII* and *sulII* only (**Figure 3** and **Table 2**). Also, one *K. pneumoniae* strain (Isolate No. 7) harboured only one resistance gene (*sulII*) and was thus excluded from the MARG assessment. Twenty-two different genotypic patterns were observed in the MDR isolates carrying more than one resistance gene. The different MARGs show that each isolate

has its own set of genes that could have been responsible for the various resistant phenotypes.

Among the four different genera considered, the *E. coli* harboured the most frequently detected resistance genes. Their higher rate of resistance than the other genera studied is attributable to their proclivity for constant possession and inclusion of ARGs, especially within the aquatic context. The ease with which ARGs can be acquired in *E. coli* has been documented in previous studies (Ahlstrom et al., 2018; Li et al., 2019).

As seen in **Figure 3**, all the isolates with multiple antimicrobial resistance genetic determinants exhibited a co-occurrence of the β-lactamases with the non-β-lactam encoding genes, except only one *K. pneumoniae* (Isolate No. 1) which harboured only two β-lactamases. The highest frequency of resistance genes detected was in an *E. coli* isolate (Isolate No. 14). It was followed by a total of fifteen various resistance genes detected in the only *E. cancerogenus* reported in this study. The detection of high ARGs in these enteric microorganisms in effluents discharged to surface water is a cause of concern. This is because some communities rely on surface water for their daily domestic activities such as bathing, and washing. Therefore, the introduction of these biological contaminants may pose a risk to public health and safety.

Various research has emphasized that contaminated water supplies are primarily liable for enhancing the dissemination of ARGs (Zhang et al., 2009; Wright, 2010; Tacão et al., 2012). The Enterobacteriaceae family, which includes common nosocomial and opportunistic pathogens as well as natural commensals of warm-blooded animals, is known to play a key function in the spread of ARGs from environmental microorganisms to other species, ultimately reaching human pathogens (Machado et al., 2009; Tacão et al., 2012).

β-lactamases Detected in Effluents of Wastewater Treatment Plants

Generally, it was found that 72.4% ($n = 21$) of the Enterobacteriaceae analyzed harboured a minimum of one of the β-lactamase resistance genes assessed. From the 92 β-lactamases detected, 70.7% ($n = 65$) corresponds to the ESBLs and carbapenemases while the remaining 29.3% ($n = 27$) belonged to the pAmpCs. *Klebsiella* spp. harboured most of the former categories with 32.3% ($n = 21$) detected, and this was keenly followed by *E. coli* at 29.2% ($n = 19$). The *Enterobacter* spp. and *Citrobacter* spp. also harboured 20% ($n = 13$) and 18.5% ($n = 12$).

For the ESBLs, the CTX-M group was found the most frequently at 21.5% ($n = 14$) with distribution as follows; $bla_{CTX-M-2\ group}$ 13.8% ($n = 9$), $bla_{CTX-M-1\ group}$ 4.6% ($n = 3$), and $bla_{CTX-M-9\ group}$ 3.1% ($n = 2$). The next dominant category of the ESBLs detected was bla_{TEM} with 20% ($n = 13$), followed by bla_{SHV} with 16.9% ($n = 11$). Other β-lactamase variants detected were bla_{IMP} with 12.3% ($n = 8$), $bla_{OXA-1-like}$ with 10.8% ($n = 7$), $bla_{OXA-48-like}$ with 6.1% ($n = 4$). The variants with the lowest frequency were detected at 3.1% ($n = 2$), and these include bla_{PER}, bla_{VIM} bla_{KPC}, and bla_{GES} (**Figure 4A**). Regarding the distribution of the β-lactamases by microorganisms, the predominance of bla_{TEM}, bla_{SHV}, $bla_{CTX-M-2\ group}$, and bla_{IMP} can be noted in all bacterial genera. A detailed breakdown of the percentages of the various genotypes detected with the corresponding number of each gene detected across the bacterial genus is presented in **Figure 4A**.
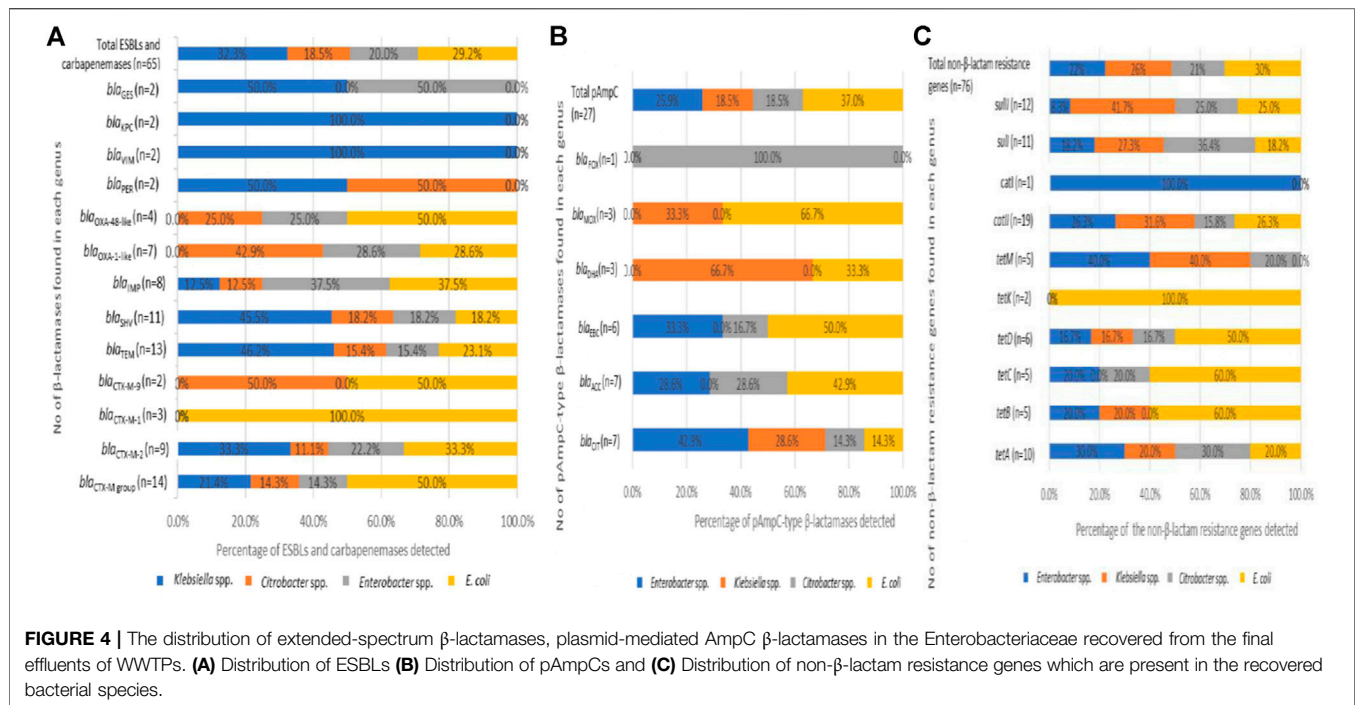
Initially, the most common β-lactamase enzyme is the TEM-1 that could hydrolyze penicillins among the Enterobacteriaceae. Soon after, the bla_{TEM} spread rapidly to other species and, with a few changes in their amino acid components, gave rise to bla_{SHV} enzymes, which hydrolyze cephalosporins. However, the bla_{SHV} were more frequently detected in *Klebsiella* spp. than the bla_{TEM} (Datta and Kontomichalou, 1965; Brunton et al., 1986; Bush and Jacoby, 2010; Aristizábal-Hoyos et al., 2019). As per this study, the highest frequency of bla_{TEM}, 46.2% ($n = 6$) and bla_{SHV}, 45.5% ($n = 5$), were recorded among the *Klebsiella* spp. (**Figure 4A**). The *Klebsiella* spp. harbouring β-lactamases such as the bla_{TEM} and bla_{SHV} have been previously reported to cause nosocomial infections (Rawat and Nair, 2010).

Since the detection of another β-lactamase enzyme, $bla_{CTX-M-1}$, recovered in an *E. coli* isolate (Bauernfeind et al., 1990; Bush and Jacoby, 2010), it has been known to spread rapidly in different bacterial species. The ability of the bla_{CTX-M} group to spread rapidly in other Enterobacteriaceae has been established

(Poirel et al., 2002; Aristizábal-Hoyos et al., 2019), so this makes the rate of detection of the bla_{CTX-M} higher than the earlier detected enzymes bla_{TEM} and bla_{SHV}. The bla_{CTX-M} group has been reported as the most predominant enzymes among the β-lactamases, followed by the bla_{TEM} (Tham, 2010; Ahlstrom et al., 2018; Aristizábal-Hoyos et al., 2019). This report is consistent with the predominant detection of the $bla_{CTX-M\ group}$ (21.5%) keenly followed by the bla_{TEM} (20%) detected in this study. Some other researchers have also described the dominance of the bla_{CTX-M} variants in many different surface water (Zarfel et al., 2013; Zurfluh et al., 2013; Ojer-Usoz et al., 2014). Also, the bla_{CTX-M} variants are among the most widespread in South Africa (Njage and Buys, 2015; Fadare et al., 2020). Interestingly, we discovered isolates from various genera such as *E. cancerogenus*, *E. cloacae*, *C. koseri*, *C. freundii*, *K. oxytoca*, *K. pneumoniae*, and *E. coli* harbouring the various bla_{CTX-M} variants. It emphasizes the significance of the research into the existence of cephalosporinases in WWTP final effluents. This study's finding of the highest frequency of bla_{CTX-M} genes in *E. coli* confirms previous findings. Also, the presence of ESBL in this genus is common, as these enzymes impart resistance to cephalosporins, one of the most commonly used antibiotic classes in clinical practice (Shi et al., 2015; Borgogna et al., 2016; Aristizábal-Hoyos et al., 2019). The bla_{TEM} gene is also one of the most frequently identified genes conferring resistance to β-lactams. It has been shown to be generally discovered in effluents of WWTPs owing to its ability to evade the wastewater treatment stages and, consequently, be detected in rivers downstream of WWTPs (Yu et al., 2018; Aristizábal-Hoyos et al., 2019). The finding of the various ESBLs variants, especially the dominance of bla_{TEM}, bla_{SHV}, and bla_{CTX-M}, in all the bacterial genera recovered from the treated final effluent of these WWTPs, is important. It appears that the traditional biological treatment used in these WWTPs selectively favours the development of antimicrobial resistance, which may have arisen on account of interactions amid susceptible and resistant microorganisms, as well as the continual selection pressure exerted by antibiotics at subinhibitory concentrations (Aristizábal-Hoyos et al., 2019).

In the other category of the β-lactamases assayed, the pAmpCs resistant gene determinants were also detected in this study. The highest frequency of the total pAmpC variants detected was harboured in *E. coli* with 37% ($n = 10$). In this category, the most predominant was bla_{CIT} and bla_{ACC} with 25.9% ($n = 7$) each. The bla_{FOX} was detected in only one *C. freundii* (Isolate No. 24), as seen in **Figure 3**. The percentage frequencies of these pAmpC-type resistance genes are as indicated in **Figure 4B**. Globally, the frequencies of the detection of the pAmpCs have been reported to be lower than their ESBL counterpart (Aristizábal-Hoyos et al., 2019). The detection of plasmid-mediated resistance genes in this study shows that they are not only restricted to the clinical settings but are also detected even in the environment.

In comparison to other studies, the prevalence of the pAmpCs reported in this study varied. In the final effluent of WWTPs in Colombia, only two categories of pAmpCs were detected in *E. cloacae, E. coli*, and *C. freundii*. Here, the incidence of LAT/BIL/

FIGURE 4 | The distribution of extended-spectrum β-lactamases, plasmid-mediated AmpC β-lactamases in the Enterobacteriaceae recovered from the final effluents of WWTPs. **(A)** Distribution of ESBLs **(B)** Distribution of pAmpCs and **(C)** Distribution of non-β-lactam resistance genes which are present in the recovered bacterial species.

CMY belonging to the CIT group was similar (n = 7) to that observed in our study, while the incidence of ACT/MIR belonging to the EBC group (n = 1) was relatively lower. Suffice to say that comparing antibiotic resistances in microorganisms across studies is a good predictor of incidence. However, it does not prove real disparities in incidence due to differences in sample forms, strain isolation methods, or resistance testing approaches (Njage and Buys, 2015). However, even a low prevalence of pAmpCs in Enterobacteriaceae is a cause for concern because clinical specimens have been shown to have elevated levels of AmpC β-lactamase expression. A significant threat is the increased development of chromosomal AmpC β-lactamases associated with the possession of plasmid-mediated AmpC-lactamases (Jacoby, 2009).

In the Enterobacteriaceae group, the principal means of antimicrobial resistance is the ability to produce β-lactamases. These enzymes break the chemical structural backbone, the β-lactam ring, of the largest and commonly used antibiotic classes (Bush and Jacoby, 2010). From the results of this study indicated in **Table 2**, these isolates' ability to possess these β-lactamases is quite concerning. This scenario implies that existing antibiotics' efficacy could be undermined while also hindering the development of new ones.

Non-β-Lactam Resistance Encoding Genes Detected in Effluents of Wastewater Treatment Plants

Plasmids carrying genes responsible for various β-lactamases usually encode resistance against other antimicrobial classes due to their large sizes (Jacoby, 2009). In this study, it was

found that 86.2% (n = 25) of the Enterobacteriaceae analyzed have a minimum of one of the non-β-lactam resistance encoding genes examined. The various ARGs against the antibiotic class tetracyclines was the most frequently detected at 43.4% (n = 33). The resistance against the sulphonamide class was 30.3% (n = 23), while the resistance against the antibiotic class phenicols was 26.3% (n = 20). The frequency of each of the non-β-lactam resistance determinants across the Enterobacteriaceae investigated includes *tetA* (13.2%), *tetD* (7.9%), *tetB, tetC,* and *tetM* (6.6% each), *catII* (25%), *catI* (1.3%), *sulII* (15.8%), and *sulI* (14.5%).

E. coli harboured the highest resistance genes detected at the genus level with 30.3% (n = 23). It was represented in almost all the categories of the genes assayed except *tetM* and *catI*. *E. coli* harboured the only *tetK* detected. The percentage frequency of detecting each of the non-β-lactam resistance genes across the various bacterial genus is shown in **Figure 4**. The high rate of detection of non-β-lactam resistance genes observed is attributable to the fact that these antibiotic classes are commonly administered as first-line antimicrobials in clinical and veterinary settings.

CONCLUSION

The study revealed the presence of MDR Enterobacteriaceae's members in the final effluents of WWTPs along with a repertoire of various important clinically relevant β-lactamases, and other essential non-β-lactam resistance genes that can be easily transferred to other species in a variety of niches. This diversity confirms the selection pressure present in the WWTPs, implying that this type of environment favours the

development and widespread of ARB and ARGs due to the dissemination of plasmid-encoded enzymes capable of hydrolyzing essential antibiotics. It also shows that, even when functioning at optimal capacity, the current design of the studied WWTPs do not enable them to prevent the spread of ARB and ARGs into the environment, posing a serious public health danger. In developing countries like South Africa, where surface water is used for domestic and irrigation purposes the poor quality of effluent discharged from WWTPs is a major concern for public health safety. Hence, this study reveals that concerted efforts should be made to improve WWTP's processes to include monitoring the presence of ARGs in wastewater before they are released into the environment. The bla_{CTX-M} genes which was widely detected in this study and other similar studies may be used as a marker to monitor the spread of resistance and mitigate the risk on human health.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.711950/full#supplementary-material

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