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Molecular Classification and Antimicrobial Profiles of Chlorination-Resistant *Escherichia Coli* at Wastewater Treatment Plant in the North West Province of South Africa

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Abstract The resistance of different pathogenic variants of *E. coli* to antibiotics, is a health concern globally. The study assessed the resistance of 90 *E. coli* isolates that survived chlorination at a Wastewater Treatment Plant (WWTP) in North West, South Africa (NW-SA), to 12 different antibiotics using the Kirby-Bauer disk diffusion method. The study further assessed the diarrheagenic pathotypes origin of the isolates. The molecular characterization revealed diarrheagenic *E. coli* pathotypes ranged as follows: Enteroaggregative *E. coli* (EAEC) 16 (17.78%), Enteroinvasive *E. coli* (EIEC) 6 (6.67%), Enterotoxigenic *E. coli* (ETEC) 5 (5.56%) and Enteropathogenic *E. coli* (EPEC) 3 (3.33%). A high degree of resistance was observed against sulphamethoxazol (92.22%), while lower resistance was observed against Kanamycin (3.33%), chloramphenicol (5.56%) and ciprofloxacin (6.67%). Multiple drug resistance of three and more antibiotics was observed in 81.11% of the *E. coli* isolates. The detected diarrheagenic *E. coli* pathotypes showed multiple resistance to different studied antibiotics with Multiple Antibiotic Resistance Indexing (MARI) equal to 0.9 for EIEC and EAEC respectively, followed by ETEC at 0.8 and EPEC at 0.2. The study reveals that the wastewater effluent from the studied plant serves as an important reservoir for the distribution of antibiotic resistant diarrheagenic *E. coli* pathotypes and other potential pathogens to the aquatic milieu, thus confirming potential risk to public health.

Keywords *E. coli* · Antibiotics · Wastewater · Diarrheagenic pathotypes · Resistance

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1 Introduction

Escherichia coli is a Gram-negative facultative anaerobe bacteria that has caught the attention of researchers since its discovery in 1885 (Tenaillon et al., 2010; Zhi et al., 2016). *Escherichia coli* presents a possible significant pathogenicity if released into the receiving water environment via inadequately treated wastewater (Osuolale & Okoh, 2017). It is for this reason that

WWTP are important facilities for treatment of wastewater to avert environmental pollution that can pose significant risk to aquatic and public health (West & Mangiameli, 2000). Wastewater Treatment Plants have the responsibility to treat industrial and domestic wastewater discharge and ensuring that microbial load in effluents from the treatment plants are significantly reduced to meet the acceptable standard. In recent years, many treatment facilities have been reported to discharge effluents contaminated with relatively high level of pathogenic microorganisms (Anastasi et al., 2012; Makuwa et al., 2020), causing a possible risk to human health.

Some communities in SA still rely on polluted surface waters that are possibly contaminated by inadequately treated wastewater effluents for their domestic needs (Jagals, 1997; Omar & Barnard, 2010). The contamination of surface water with sewage waste can be monitored through the detection of *E. coli* and fecal coliform bacteria that serve as indicators of the presence of disease causing microorganisms (Tallon et al., 2005; Young & Thackston, 1999). Most strains of *E. coli* are harmless, but some are pathogenic and known to cause variety of gastrointestinal and extraintestinal disease (Kaper et al., 2004; Nataro & Kaper, 1998; Xia et al., 2011). To date, there are several studies that have reported the identification of intestinal pathogenic *E. coli* (IPEC) or diarrheagenic *E. coli* (DEC) groups from WWTP effluents. The pathotypes in this regard, include enteropathogenic *E. coli* (EPEC), enterohaemorrhagic/Shiga-toxin producing *E. coli* (EHEC)/STEC, enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) (Croxen & Finlay, 2010; Nataro & Kaper, 1998; Omar & Barnard, 2010; Robins-Browne et al., 2016; Shabana et al., 2013) and extraintestinal *E. coli* (ExPEC) (Köhler & Dobrindt, 2011; Russo & Johnson, 2000; Xia et al., 2011). Diarrheagenic *E. coli* is the main cause of worldwide epidemic and endemic diarrhea (Bonkoungou et al., 2012; Kaper et al., 2004; Shetty et al., 2012). Extraintestinal *E. coli* strains cause infections of any organ or anatomical site due to specialized virulence factors that are known to cause a broad spectrum of diseases and are not present on commensal *E. coli* (Kaper et al., 2004; Russo & Johnson, 2000).

Previous studies have shown an increase in antibiotic resistance bacteria from WWTPs effluents and that had recently led to a global concern (Dolejska et al., 2011; Redhead et al., 2020; Rizzo et al., 2013; Vaz-Moreira et al., 2014). Wastewater treatment facilities serve as a primary water reservoir and key potential gateways for antibiotic-resistant bacteria including *E. coli*'s of human and animal origin interfacing within the aquatic environment (Dolejska et al., 2011; Martinez, 2009; Osuolale & Okoh, 2017). The inadequate treatment of wastewater by treatment facilities often introduces pathogens and antibiotic-resistant *E. coli* into natural water resources (Rizzo et al., 2013; Vaz-Moreira et al., 2014), escalating the risk of infection (Dolejska et al., 2011; Igwaran et al., 2018; Ivanov et al., 2005; Martinez, 2009; Osuolale & Okoh, 2017). Bacterial populations received by wastewater treatment facilities from diverse sources interact and exchange antibiotic-resistant genes horizontally (Arana et al., 2001; Igwaran et al., 2018; Karkman et al., 2018). As an example, there are reports on *Escherichia coli* from WWTP effluents that have been shown to be resistant to several number of medically significant antibiotics (Abdul et al., 2013; Buvens et al., 2010; Osuolale & Okoh, 2017).

The treatment facility in this study was recently reported to discharging effluents contaminated with chlorine-disinfection resistant *E. coli* (Makuwa et al., 2020). The aim of the currently study is therefore, to assess the pathogenicity and antibiotic resistance profiles of the *E. coli* strains at the WWTP. Studying the antimicrobial resistance pattern of the pathogenic *E. coli* strains, is noteworthy in order to determine the shift in antibiotic resistance patterns among the pathogens and to adapt control measures that will help prevent the discharging of pathogenic multidrug-resistant *E. coli* strains to the environment.

2 Materials and Method

2.1 Study area

The plant of interest in this study (Coordinates: latitude: -26.75141 longitude: 27.0945) is situated in the North West Province of South Africa (NW-SA). The town where the plant is situated has a population of about 124,000 and it is an industrial and agricultural area for North West Province (Makuwa et al., 2020). The plant therefore receives municipal domestic sewage and wastewater that is heavily influenced

by household and industrial water use. The plant is an activated sludge treatment plant. The treatment of physicochemical impurities is done through preliminary, primary, and secondary stages. The secondary stage operates through the Phoredox and Bardenpo activated sludge configurations.

There are different disinfection processes for treatment of wastewater in SA, of which chlorination is the most commonly applied (Bekink & Nozaic, 2013; Virto et al., 2005; Yang & Zhang, 2013). The plant studied uses chlorine gas as disinfectant. A dosing of 10 kg of chlorine/h is applied across all seasons. The studied plant uses chlorination as a form of disinfection. The contact time for disinfection at the tertiary treatment stage is 30 min.

2.2 Sample collections

A total of 90 WWTP final effluent samples were collected aseptically at the final discharge point using sterile 250 mL sampling bottles for the analysis of *E. coli*. The final discharge point is a point situated after disinfection process before treated wastewater enter the environment. The sampling containers were washed with soap and water and autoclaved after each use. Samples were collected on a weekly bases, between May 2019 and March 2020.

2.3 Isolation and confirmation of presumptive *E. coli*

The Colilert Quanti-Tray/2000 system as described in Omar et al., (2010) was used for the enumeration of the viable *E. coli* cells from the 90 samples studied. Enumeration of *E. coli* from samples was done by using 100 mL water according to the manufacturer's instructions. The Quanti-Trays were incubated for 18–22 h at 37 ℃. After incubation, the Quanti-Trays/2000 were examined under long wave (366 nm) ultraviolet light, and wells that turned both yellow and fluoresced were counted as *E. coli* positive (IDDEX). The results of the quantifications were reported as *E. coli* count/100 mL. To get colonies for DNA extraction, isolates from the fluorescence wells of the Colilert Quanti-Tray/2000 system were sub-cultured on Eosin Methylene Blue agar (EMB agar) (Merck, Germany) and incubated at 37 °C for 24 h. Colonies of *E. coli* isolates were confirmed by a distinctive metallic green sheen appearance on EMB agar.

2.4 DNA extraction

Two colonies of pure isolated bacteria were placed into a tube containing 100 µL of double distilled water. The tubes were heated at 100 °C for 10 min, and then the cells were pelleted by centrifugation. The supernatant containing DNA was taken out and stored at -20 °C (Kazemnia et al., 2014; Obeng et al., 2012).

2.5 Molecular confirmation of the different diarrheagenic pathotypes

The confirmed isolates were delineated by using PCR into different *E. coli* diarrheagenic pathotypes based on the presence of virulence genes in their genome according to Tanih et al., (2015). The list of the studied diarrheagenic genes were categorised based on their functional characteristics (see Table 1). The diarrheagenic pathotypes of the confirmed *E. coli* isolates were determined with the aid of PCR technique using of specific primers targeting *LT* and *ST* genes for ETEC, *stx1* and *stx2* genes for EHEC, *eae* and *bfpA* genes for EPEC, *ipaH* gene for EIEC, *aaTA* and *aaic* genes for EAEC, as well as *daaE* gene for DAEC, as listed in Table 2.

Multiplex polymerase chain reaction analysis of the targeted genes of interest was performed using DreamTaq DNA polymerase (Thermo Scientific, USA). For the amplification, 5 µL of DNA was added to 20 µL of master mix containing 12.5 µL of DreamTaq DNA polymerase (2X DreamTaq Green Buffer, dATP, dCTP, dGTP, and dTTP, 0.4 mM each, and 4 mM $MgCl_2$) (Thermo Scientific, USA), 0.5 µL (0.2 µM) of respective oligonucleotide primers. The reaction volume was made up with nuclease free water. PCR was performed in a thermal cycler (Bio-Rad Laboratories, USA). The reactions were subjected to an initial activation step at 95 °C for 15 min, followed by 35 cycles consisting of denaturing at 94 °C for 45 s, annealing at 55 °C for 45 s, extension at 68 °C for 2 min and final elongation at 72 °C for 5 min (Omar & Barnard, 2010). The primers used to amplify the targeted genes were as previously reported by Tanih et al., (2015) and Igwaran et al., (2018) as detailed in Table 2. Negative controls, substituting DNA template with ultrapure water (Sigma-Aldrich, UK), were included in all PCR runs. DNA extracted from *E. coli* ATCC 25922 was used as a

Table 1 Categorisation of studied diarrheagenic genes based on their functional characteristics and association with *E. coli* pathotypes (Osuolale, 2015)

Diarrheagenic *E. coli* pathotypes	Adhesion gene	Toxin gene	Invasion gene	Function
ETEC		*LT* *ST*		Heat-labile toxin
EHEC		*stx1* *stx2*		Shiga-toxin 1 and 2
EPEC	*eae* *bfpA*			Attaching and effacing
EIEC			*ipaH*	Invasion plasmid antigen
EAEC	*aatA* *aaic*			Transcriptional regulator for chromosomal gene/Enteroaggregative adhesion
DAEC	*daaE*			

Table 2 Primer sequences used for detection *E. coli* pathotypes

E. coli & Pathotypes	Primer sequences	Product Size (kb)	References
ETEC (*LT*)	**F-** 5'-CACACGGAGCTCCTCAGTC-3' **R-** 5'-CCCCCAGCCTAGCTTAGTTT-3'	508 bp	Tanih et al., 2015)
ETEC (*ST*)	**F-** 5'-GCTAAACCAGTAGAGGTCTTCAAAA-3' **R-** 5'-CCCGGTACAGAGCAGGATTACAACA-3'	147 bp	Tanih et al., 2015)
EHEC (*Stx1*)	**F-** 5'-CAGTTAATGTGGTGGCGAAGG-3' **R-** 5'-CACCAGACAATGTAACCGCTG-3'	384 bp	Tanih et al., 2015)
EHEC (*Stx2*)	**F-** 5'-ATCCTATTCCCGGGAGTTACG-3' **R-** 5'-GCGTCATCGTATACACAGGAGC-3'	584 bp	Tanih et al., 2015)
EPEC (*eae*)	**F-** 5'-CCCGAATTCGGCACAAGCATAAGC-3' **R-** 5'-CCCGGATCCGTCTCGCCAGTATTCG-3'	881 bp	Tanih et al., 2015)
EPEC (*bfpA*)	**F-** 5'-GGAAGTCAAATTCATGGGGGTAT-3' **R-** 5'-GGAATCAGACGCAGACTGGTAGT-3'	300 bp	Tanih et al., 2015)
EIEC (*ipaH*)	**F-** 5'-TGGAAAAACTCAGTGCCTCT-3' **R-** 5'-CCAGTCCGTAAATTCATTCT-3'	423 bp	Tanih et al., 2015)
EAEC (*aatA*)	**F-** 5'-CTGGCGAAAGACTGTATCAT-3' **R-** 5'-CAATGTATAGAAATCCGCTGTT-3'	650 bp	Tanih et al., 2015)
EAEC (*aaic*)	**F-** 5'-ATTGTCCTCAGGCATTTCAC-3' **R-** 5'-ACGACACCCCTGATAAACAA-3'	215 bp	Tanih et al., 2015)
DAEC (*daaE*)	**F-**5'-GAACGTTGGTTAATGTGGGGTAA-3' **R-**5'-TATTCACCGGTCGGTTATCAGT-3'	542 bp	Igwaran et al., 2018)

positive control. Amplified DNA was resolved by 2% agarose gel electrophoresis and visualised under UV transillumination.

2.6 Antibiotic resistance testing

The resistance/susceptibility testing of all *E. coli* isolates was performed using the Kirby-Bauer disk diffusion method as described in the study by Kazemnia et al., (2014) and Osuolale and Okoh, (2017). A volume of 100 µL of an overnight growth *E. coli* isolate on Nutrient broth with 0.5 McFarland standard turbidity was streaked on Mueller- Hinton agar plates (Conda, Madrid). The study used 12 antibiotic discs, all from HiMedia® (India). The antibiotics included: cephazolin, gentamicin, ciprofloxacin, streptomycin, trimethoprim, amoxycillin, neomycin, kanamycin, chloramphenicol, sulphamethoxazol, nalidixic

acid and tetracycline. The choices of antibiotic panels selected were based upon the recommendation of CLSI (CLSI, 2012). The group arrangements of these studied antibiotics were as follows: Cephems (cephazolin), Aminoglycosides (gentamicin, streptomycin, neomycin and kanamycin), Quinolones and Fluoroquinolones (ciprofloxacin and nalidixic acid), Folate Pathway Antagonists (trimethoprim and sulphamethoxazol), β-Lactam Combination Agent (amoxicillin), Phenicols (chloramphenicol), and Tetracyclines (tetracycline) (see Table 3). The antibiotics discs were placed on the surface of the inoculated Mueller-Hinton agar plates. After 10 min at room temperature, the plated cultures were incubated in an inverted position at 37 °C for 20–24 h. The zones of inhibition were measured and compared with standard chart.*E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as antibiotic controls. Isolates with intermediate resistance were defined as susceptible, and the isolates were considered as multidrug resistant if they were resistant to at least three classes of antibiotics (Bashir et al., 2011; Blanco et al., 2011; Bukh et al., 2009; Kazemnia et al., 2014).

2.7 Multiple Antibiotic Resistance Indexing (MARI)

Multiple antibiotic resistant (MAR) phenotypes were generated for strains that showed resistance to three or more antibiotics. MAR index was calculated as previously described by Osuolale, (2015) and is mathematically expressed as:

$$MARI = a/b$$

where

a *number of antibiotics to which the isolate was resistant;*

b *total number of antibiotics against which individual isolate was tested.*

Table 3 Classification of antibiotics according to their chemical grouping

CHEMICAL GROUPS	EXAMPLES OF ANTIBIOTICS
Cephems	Cephazolin KZ30
Aminoglycosides	Gentamicin CN10
	Streptomycin S10
	Neomycin N30
	Kanamycin K30
Quinolones and Fluoroquinolones	Ciprofloxacin CIP5
	Nalidixic Acid NA30
Folate Pathway Antagonists	Trimethoprim W5
	Sulphamethoxazol RL100
β-Lactam Combination Agent	Amoxycillin AML10
	Ampicillin AMP10
Phenicols	Chloramphenicol C30
Tetracyclines	Tetracycline TE30

3 Results

3.1 Molecular characterization of *E. coli* isolates

A total of 90 presumptive *E. coli* isolates were obtained from WWTP final effluent samples through Colilert Quanti-Tray/2000 (Omar et al., 2010). The 90 presumptive *E. coli* isolates recovered from the fluorescent Quanti-tray wells, were further confirmed by their distinctive metallic green sheen appearing on the surface of the bacterial colonies on EMB agar (Leininger et al., 2001). Among the 90 confirmed *E. coli* isolates assessed for the various diarrheagenic *E. coli* genes, 32 (35.56%) harbored at least 1 or more virulent genes while 58 (64.44%) isolates harbored none. The outcome of the different diarrheagenic pathotypes from *E. coli* isolates as indicated in Table 4, showed positive detection of ETEC 5 (5.56%), EPEC 3 (3.33%), EIEC 6 (6.67%), and EAEC 16 (17.78%) from the six studied diarrheagenic pathotypes, while no detections were observed for EHEC and DAEC *E. coli* pathotypes.

The diarrheagenic *E. coli* genes were categorised into toxin, adhesion and invasion genes based on functional characteristics of the genes as indicated in Table 1. Such categorisation enabled the study to identify the prevalence of these virulence genes with observable differences to each sample. The targeted genes were as follows: ETEC (LT and ST), EHEC (stx1 and stx2), EPEC (eae and bfpA), EAEC (aatA and aaic), EIEC (ipaH) and DAEC (daaE). The distribution of the targeted genes as presented in Table 4, showed *aatA* and *aaiC* genes dominating with 9 (10%) and 8 (8.89%) positive

Table 4 Distribution of the diarrheagenic *E coli* pathotypes and the targeted genes

Pathotypes	No. of positive isolates / (%)	Targeted Genes	No. of positive genes / (%)	No. of shared genes on same isolates / (%)
ETEC	5 (5.56)	*LT*	1 (1.11)	1 (20)
		ST	5 (5.56)	
EHEC	0 (0)	*stx1*	0 (0)	0 (0)
		stx2	0 (0)	
EPEC	3 (3.33)	*Eae*	3 (3.33)	0 (0)
		bfpA	0 (0)	
EAEC	16 (17.78)	*aatA*	9 (10)	1 (6.25)
		Aaic	8 (8.89)	
EIEC	6 (6.67)	*ipaH*	6 (6.67)	N/A
DAEC	0 (0)	*daaE*	0 (0)	N/A

isolates, respectively. Other diarrheagenic pathotypes genes were variously detected as follows; *ipaH* 6 (6.67%), *ST* 5 (5.56%), *eae* 3 (3.33%) and *LT* 1 (1.11%). Of the 5 positive ETEC isolates shown in Table 4, 1 (20%) isolate presented both of *ST* and *LT* genes, while similar results of single isolates (6.25%) were also observed with genes (*aatA* and *aaic*) meant to confirm the presence of EAEC among the 16 detected isolates. Both EHEC and EPEC did not show shared genes amongst their isolates, while EIEC and DAEC isolates were detected using single genes primers. The representative gel electrophoresis profiles of amplified products of the investigated diarrheagenic *E. coli* virulence genes are shown in Fig. 1.

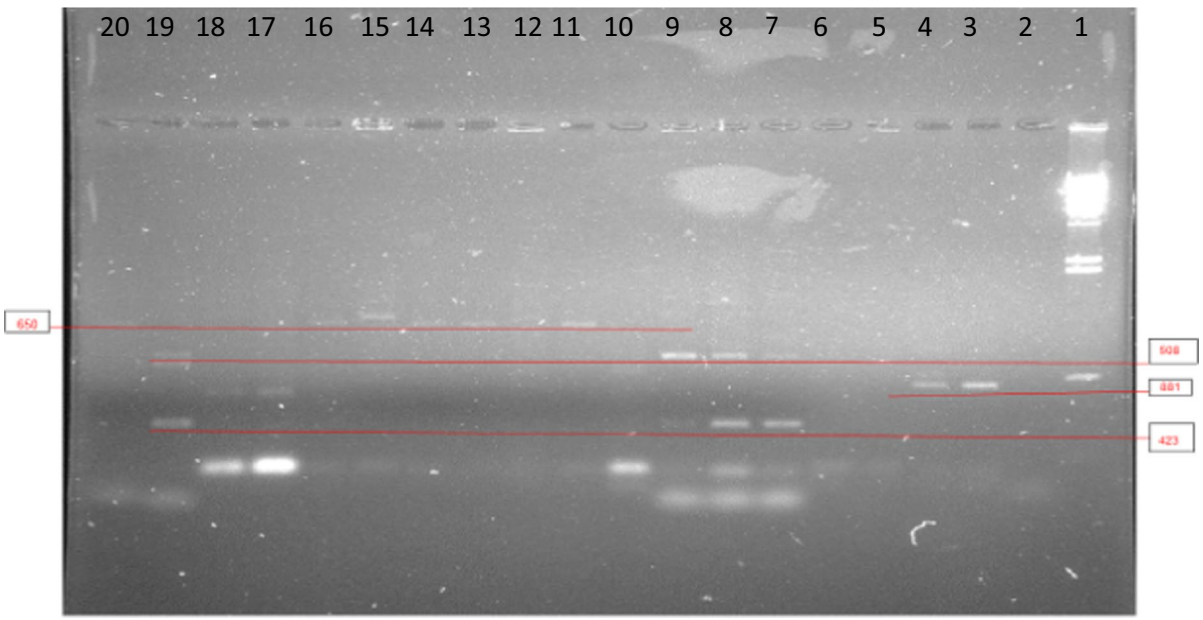

Fig. 1 A representative gel electrophoresis profile of different virulence genes of isolated *E. coli*. Lane 1: molecular weight marker (Merck 1 kb DNA ladder), lane 2: negative control, lane 3: *LT* (508 bp), lane 4: *LT* (508 bp), lane 5: negative test, lane 6: negative test, lane 7: *aatA* (650 bp), lane 8: *aatA* (650 bp), lane 9: *aatA* (650 bp), lane 10: negative test, lane 11: *eae* (881 bp), lane 12: negative test, lane 13: *eae* (881 bp), lane 14: *eae* (881 bp), lane 15: *eae* (881 bp), lane 16: *eae* (881 bp), lane 17: *ipaH* (423 bp), lane 18: *ipaH* (423 bp), lane 19: *aatA* (650 bp), lane 20: negative control

3.2 Antibiotic resistance profiling of confirmed *E. coli* isolates

A total of 12 antibiotics were assessed against the 90 *E. coli* isolates extracted from WWTP final effluent samples. The presence of *E. coli* in the final effluent confirmed their survival to chlorination which is the disinfection method applied at the studied plant. The antibiotics included: cephazolin, gentamicin, ciprofloxacin, streptomycin, trimethoprim, amoxicillin, neomycin, kanamycin, chloramphenicol, sulphamethoxazol, nalidixic acid and tetracycline.

The antibiotic susceptibility, intermediate and resistant profiles of the *E. coli* isolates are presented in Fig. 2. The *E. coli* isolates were mostly resistant to sulphamethoxazol, with less resistance observed against Kanamycin (3.33%), chloramphenicol (5.56%) and ciprofloxacin (6.67%). The overall resistance profiles of the *E. coli* isolates were as follows: sulphamethoxazol (92.22%), tetracycline (56.67%), trimethoprim (52.22%), neomycin (48.89%), nalidixic acid (41.11%) streptomycin (40%), amoxicillin (40%), cephazolin (37.78%), gentamicin (12.22%), ciprofloxacin (6.67%), chloramphenicol (5.56%) and kanamycin (3.33%). Multi-antibiotic resistance was considered when the isolate was resistant to three and more antibiotics with 81.11% of multi drug resistance cases observed.

3.3 Antibiotic resistance profiling of confirmed diarrheagenic *E. coli* pathotypes

The antibiotic profiles of the studied diarrheagenic *E. coli* pathotypes are shown in Table 5. Both EIEC and EAEC showed multiple resistance to all the studied antibiotics, except for Kanamycin (EIEC) and Chloramphenicol (EAEC) respectively. The least resistance was observed in EAEC with regard to Kanamycin (6%). EIEC (0.9), EAEC (0.9) and ETEC (0.8) showed highest MAR index in relation to antiobiotic resistance, while EPEC (0.2) showed the least MAR index.

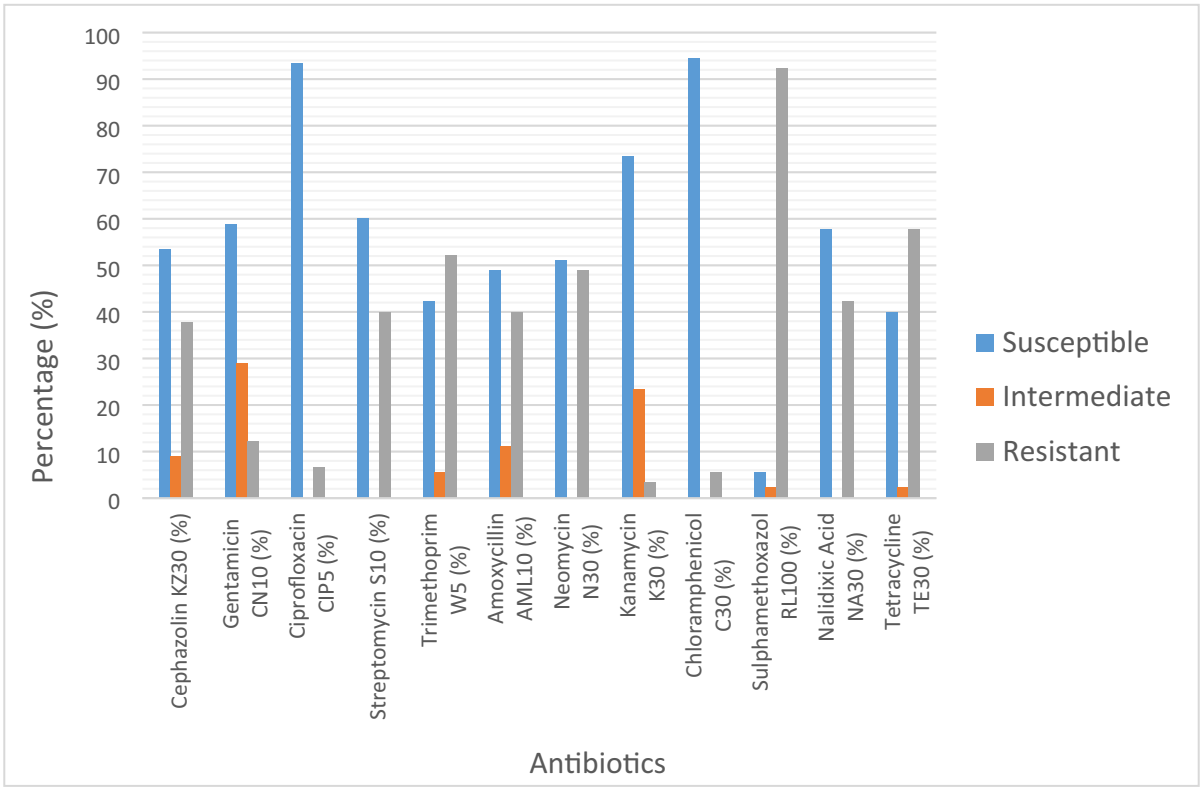

Fig. 2 Antibiotic susceptibility, intermediate and resistant profiles of the *E. coli* isolates

Table 5 Summary of antimicrobial resistance determinants among diarrheagenic *E. coli* pathotypes

Antibiotics	Diarrheagenic *E. coli* Pathotypes					
	ETEC $n=5$	EHEC $n=0$	EPEC $n=3$	EIEC $n=6$	EAEC $n=16$	DAEC $n=0$
Cephazolin KZ30	2(40%)	0(%)	0(%)	2(33%)	2(13%)	0(%)
Gentamicin CN10	0(%)	0(%)	0(%)	1(17%)	2(13%)	0(%)
Streptomycin S10	2(40%)	0(%)	0(%)	3(50%)	9(56%)	0(%)
Neomycin N30	4(80%)	0(%)	2(67%)	2(33%)	9(56%)	0(%)
Kanamycin K30	0(%)	0(%)	0(%)	0(%)	1(6%)	0(%)
Ciprofloxacin CIP5	1(20%)	0(%)	0(%)	2(33%)	2(13%)	0(%)
Nalidixic Acid NA30	2(40%)	0(%)	0(%)	5(83%)	6(38%)	0(%)
Trimethoprim W5	1(20%)	0(%)	0(%)	4(67%)	6(38%)	0(%)
Sulphamethoxazol RL100	4(80%)	0(%)	3(100%)	6(100%)	13(81%)	0(%)
Amoxycillin AML10	1(20%)	0(%)	0(%)	3(50%)	4(25%)	0(%)
Chloramphenicol C30	0(%)	0(%)	0(%)	2(33%)	0(%)	0(%)
Tetracycline TE30	3(60%)	0(%)	0(%)	6(100%)	5(31%)	0(%)
MARI	**0.8**	**0**	**0.2**	**0.9**	**0.9**	**0**

4 Discussion

Escherichia coli is commonly known as an indicator that predict possible presence of other pathogens of enteric origins (Cabral, 2010; Jamieson et al., 2002; Motlagh & Yang, 2019; Rompré et al., 2002). This organism underlines the importance of municipal WWTPs as potential point sources of pathogens into environmental waters (Adefisoye & Okoh, 2016). In this study, the prevalence, and the antibiotic resistance profiling of diarrheagenic *E. coli* remoted from NW-SA WWTP final effluent samples, were investigated. Diarrheagenic *E. coli* are primary etiological agents of pediatric diarrhea, which remains the most common cause of infantile morbidity and mortality especially in developing countries. The organisms are transmitted through the oral-fecal path by ingesting food or water contaminated by human or animal feces (Adefisoye & Okoh, 2016).

Diarrheagenic *E. coli* are the principal cause of demise globally, especially in developing nations (Bryce et al., 2005; Igwaran et al., 2018; Shabana et al., 2013). Amongst the six diarrheagenic *E. coli* pathotypes profiled from the 90 confirmed *E. coli* isolates, only ETEC, EPEC, EAEC, and EIEC were detected, while none of the isolates showed targeted virulence genes for EHEC and DAEC. These pathotypes were identified based on the targeted genes shown in Tables 1 and 2, however contrary to the study by Igwaran et al., (2018), *daaE* gene for DAEC was not identified in this study. The presence of these diarrheagenic *E. coli* pathotypes in the environment calls for concern due to their public health concerns (Clements et al., 2012; Haller et al., 2009). The detected diarrheagenic *E. coli* pathotypes implies that the studied WWTP serves as a reservoir of the diarrheagenic *E. coli* pathotypes. Studies by Adefisoye and Okoh, (2016) and Omar and Barnard, (2010) in EC-SA and Gauteng-SA respectively, detected the presence of diarrheagenic *E. coli* from the final effluent of wastewater treatment plant and this also substantiated our findings. Of the 90 confirmed *E. coli* isolates tested, EAEC represented about 17.78% of isolates, followed by EIEC at 6.67%, ETEC at 5.56% and EPEC at 3.33%. In similar studies by Mbanga et al., (2020) and Omar and Barnard, (2010), they reported a high detection of EAEC in their treated final effluents samples. In the Eastern Cape and Limpopo Provinces of South Africa, these diarrheagenic *E. coli* pathotypes have been isolated from diarrhea patients, with EAEC being the predominant cause of infection (Bisi-Johnson et al., 2011; Samie et al., 2007). A single isolate each for ETEC and EAEC showed the presence of both targeted genes. Similar observation of shared genes (*LT* and *ST*) for ETEC isolate, were observed in previous studies by Bolukaoto et al., (2021) and Hossain et al., (2021). According to Johura et al. (2017), the genes that encode both *LT* and *ST* enterotoxins in ETEC are generally found on plasmids, transmissible and causing severe

diarrhea. The EAEC gene coding for both aaic and aatA, were also observed at a higher rate than this study from children in Norh-eastern Brazil (Lima et al., 2013).

Antimicrobial resistance testing is a famous global standard allowing laboratories to help clinicians in treating infections caused by microbial agents (Igwaran et al., 2018; Rizzo et al., 2013). The prevalence of antimicrobial resistant bacteria in WWTP effluents is a foremost public health concern (Mukherjee et al., 2021; Rodriguez-Molina et al., 2019). Effluents from WWTP used for irrigation water for crops can stimulate the distribution of antibiotic resistance genes into soils and could by some means find their ways into human system (Wang et al., 2014). The final effluent of WWTPs has been identified by studies as a prime vehicle of antibiotics resistant pathogens into the aquatic environment (Igwaran et al., 2018; Osunmakinde et al., 2019; Zerva et al., 2021).

From the confirmed *E. coli* isolates that were tested against a panel of 12 commercial antibiotics, the isolates divulged distinct resistance patterns against the antibiotics. The *E. coli* isolated from the discharged effluent, survived chlorine disinfection. With the *E. coli* isolates having survived chlorine disinfection, the antibiotic-resistant bacteria also demonstrated the capability for re-growth to a chlorine disinfection of up to 5 mg/L in a study by Destiani and Templeton, (2019). According to Huang et al., (2011), some antibiotic-resistant bacteria have been reported to demonstrate resistance to chlorine. Sulphamethoxazole and tetracycline was the highest-level resistance antibiotic, this finding corroborate the results from a study by Osuolale, (2015). Osuolale, (2015), associated the high resistance level of sulphamethoxazole to domestic, industrial and health facility wastes, surface runoff and various anthropogenic activities.

Antibiotic resistance, particularly multidrug resistance, is a major public health threat and is an emerging concern around the world (Watkinson et al., 2007). About 81.11% of the isolates showed resistance to more than three antibiotics. Adefisoye and Okoh, (2016) observed lower multidrug of 32.7% compared to this study. Multidrug resistance of *E coli* isolates was observed in 85.11% of the hospital wastewater and 73.53% of the community wastewater studied by Gaşpar et al., (2021). With *E. coli* being the most commonly studied bacteria, the resistance to at least two classes of antimicrobial agents has been regularly detected in the environment (Baum & Marre, 2005; Young, 1993). The current study revealed multidrug resistance to numbers of antibiotics ranging from 3 to 10, while Osuolale, (2015) reported multidrug ranging between 3 to 9 antibiotics. The antibiotic resistance patterns of the *E. coli* isolates were as follows: no-antibiotic resistance (0), single-antibiotic resistance (1), two-antibiotic resistance (7), three-antibiotic resistance (11), four-antibiotic resistance (13), five-antibiotic resistance (15), six-antibiotic resistance (13), seven-antibiotic resistance (18), eight-antibiotic resistance (8), nine-antibiotic resistance (3) and ten-antibiotic resistance (1). The antibiotic resistance patterns in the study by Osuolale, (2015) was as follows: no-antibiotic resistance (4), single-antibiotic resistance (36), two-antibiotic resistance (25), three-antibiotic resistance (19), four-antibiotic resistance (21), five-antibiotic resistance (24), six-antibiotic resistance (20), seven-antibiotic resistance (6), eight-antibiotic resistance (13) and nine-antibiotic resistance (5). According to the study by Murray et al., (1984), wastewater effluent disinfection has also been shown to increase the prevalence of antibiotic resistant bacteria and multidrug resistance.

Infections triggered by diarrheagenic *E. coli* pathotypes are treated with antibiotics; however, the emergence of resistant strains may affect the treatment of some infections (Ishii & Sadowsky, 2008). All the detected diarrheagenic *E. coli* pathotypes presented higher level of resistance to Sulphamethoxazol. Study by Osuolale and Okoh, (2017), showed higher resistance of isolated pathogens to tetracycline. EPEC and EIEC showed total resistance to Sulphamethoxazol. EIEC was the only diarrheagenic *E. coli* pathotype that completely showed resistance to Tetracyline. A study by Osuolale, (2015), revealed that, all their detected pathotypes showed total resistance to sulphamethoxazole and display a significant high resistance to ampicillin, amoxycillin, gentamycin, cefuroxin, tetracycline and chloramphenicol. In the study by Torres, (2009), EPEC was the common pathotype associated with multiple antibiotic resistances, while in other studies they were EPEC and ETEC (Oliveira et al., 2012), EAEC and EPEC (Hamelin et al., 2006). This study used MARI to estimate health threat related to the spread of antibiotic resistance in the environment. According to MARI calculation, the EIEC and EAEC showed highest multiple antibiotic resistance of 0.9, each, respectively, followed by

ETEC (0.8) and EPEC (0.2). According to Christopher et al., (2013), MARI above 0.2 suggests that a strain(s) of bacteria originate from an environment with excessive contamination or antibiotics usage. The high MARI values observed with EIEC, EAEC and ETEC diarrheagenic *E. coli* pathotypes acquired in this study may advocate the exposure of the pathotypes to antibiotics pressure, which may have resulted from wrong use of antibiotic among the populace of the studied area and may lead to further increase in the development of multidrug resistance if proper processes are not applied. The resistance of the diarrheagenic *E. coli* pathotypes to studied antibiotics serves as a pointer to the possible presence of other *E. coli* pathotypes inclusive of other bacterial pathogens presenting resistance to several antibiotics. The findings of this study is in line with other reports on the detection of more than one antibiotic resistance through commensal and pathogenic strains of *E. coli* (Bailey et al., 2010; Karczmarczyk et al., 2011).

5 Conclusion

The mandate of a WWTP is to notably reduce microbial constituency before the plant effluent is discharged into the environment, however vast quantities of pathogenic antibiotic resistant bacteria escape the treatment process into aquatic milieu. The confirmation of the presence of *E. coli* from WWTP final effluent in NW-SA, indicates fecal contamination and the feasible presence of other enteric pathogens inclusive of different *E. coli* pathotypes. Our findings suggest an excessive prevalence of antimicrobial resistance of diarrheagenic *E. coli* pathotypes towards the conventionally used antibiotics, thus a presenting public health risk. It is therefore significant for the regulators to review their handling of wastewater and antibiotics wastes to lessen their environmental impacts, and public health concerns.

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Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

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