

Full Length Research Paper

Isolation and antibiotic resistance profiles of bacteria from influent, effluent and downstream: A study in Botswana

K. Tapela and T. Rahube*

Department of Biological Sciences and Biotechnology, Faculty of Science, Botswana International University of Science and Technology, Botswana.

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Antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) have been globally considered environmental contaminants that pose a serious problem to the health of humans, animals and the ecosystem. The primary objective of the study was to characterize the antibiotic resistance phenotypes and genotypes of bacterial isolates from Gaborone wastewater treatment plant (GWTP) and the downstream environment receiving effluent wastewater. Culture dependent and independent approaches were used to determine occurrence and diversity of ARGs in viable and potentially pathogenic bacteria from samples of wastewater influent, effluent and downstream environment. Higher frequencies of potentially pathogenic ARB; *Staphylococcus* species, *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas* species, *Brucella* species, *Salmonella* species, *Listeria* species and *Campylobacter* species, and ARGs to clinically relevant antibiotics; *tetA* (tetracycline), *mphA* (macrolides), *strB* (streptomycin), *su1* (sulphonamide), *dfp* (trimethoprim) and *int1* (mobile ARG cassette) were detected from the samples. Taken together, the results suggest accumulation of these antibiotic resistance determinants in wastewater treatment facilities and subsequent release into the water ecosystems downstream of the WWTP. This research is critical in Botswana because of lack of data and awareness on the threat posed by antibiotic resistance, poor wastewater treatment infrastructure, and lack of policies/guidelines on the safe use/handling of effluent wastewater for agricultural purposes. Data from this research will help sensitize relevant government health officials to carefully consider the environment contamination and spread of antibiotic resistance. This study further advocates for development of new water quality monitoring schemes and implementation of locally relevant policies on the safe and sustainable use of effluent contaminated water particularly for irrigation purposes in many developing countries.

Key words: Antibiotic resistant bacteria, antibiotic resistance genes, wastewater, public health.

INTRODUCTION

Wastewater treatment plant (WWTP) is an important reservoir for development and emergence of antibiotic resistant bacteria (ARB) due to the high concentration of chemicals including antibiotic residues from different sources such as homes, abattoirs, clinics and industries.

Wastewater treatment plants (WWTPs) are interfaces between different environments (clinical and non-clinical), thus providing an opportunity for antibiotic resistance genes (ARGs) in mobile genetic elements (For example plasmids and integrons) to mix and transfer between

pathogenic and non-pathogenic environmental bacteria (Rahube and Yost, 2010). Wastewater contains many pathogenic bacterial species including the Enterobacteriaceae (*Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa*), Mycobacteriaceae (*Mycobacterium tuberculosis*) (Ye and Zhang, 2013) and Vibrionaceae (*Vibrio fluvialis*) (Ramamurthy et al., 2014). Many of the organisms present in wastewater are fast growing bacteria that have the capacity to adapt and become important vectors of ARGs (McGowan, 2006). Many bacterial species resistant to different clinically important antibiotics have been detected in wastewater influent, effluent and downstream environments (Zhang and Li, 2011; Peña-Miller et al., 2015; Keraita and Drechsel, 2004).

Many developing countries often have poor and ineffective wastewater treatment infrastructures, characterized by high inflow of raw sewage into WWTPs with low receiving capacity. Most of these treatment facilities do not have the tertiary treatment stage, and rely mostly on biological treatment. Gaborone wastewater treatment plant (GWWTP) in Botswana has been reported to collect more raw sewage than its holding capacity. Its current inflows have been estimated to be fifty-five million litres per day instead of forty million litres per day. The government of Botswana has been on the plan of upgrading GWWTP to 65 ml per day (Mguni, 2010). This situation of GWWTP means that effluents are discharged into the environment without proper or sufficient treatment. In addition, despite advances in wastewater treatment methods used mostly in developed countries, none of the current methods completely destroy all the bacteria or mobile genetic elements capable of passing and spreading antibiotic resistance (Asfahl and Savin, 2012). Therefore, downstream environments receiving the effluents remain very important sources for further accumulation and proliferation of antibiotic resistance determinants (Baquero et al., 2008; Rahube and Yost, 2010). In some parts of Africa, it has been reported that large volumes of untreated wastewater are released from WWTPs which adversely affect water bodies such as rivers, streams and ponds in both urban and peri-urban areas. The major health concern is also that farmers specifically prefer using these effluent contaminated water bodies as sources of water for irrigation. Studies have revealed high levels of microbiological contamination with antibiotic-resistant coliforms and pathogenic bacteria including the ARGs in wastewater and sludge used for agricultural purposes (Keraita and Drechsel, 2004; Zhang and Zhang 2011; Rahube et al., 2014a; Peña-Miller et al., 2015).

The World Health Organization (WHO), Europe and

the United States Centre for Disease Control and prevention (CDC) have highlighted the importance of studying the emergence and spread of antibiotic resistance as well as the need for control strategies to minimize the development and spread of antibiotic resistance to the general public. The present study focused primarily on determining the non-clinical environment occurrence, diversity and potential spread of ARB and ARGs in GWWTP and Notwane River receiving effluent wastewater. There is currently no data in Botswana on antibiotic resistance occurrence in the environment. By taking into consideration the socio-economic and ecological factors, evidence from this study will help evaluate the extent of antibiotic resistance threat in Botswana and provide recommendations on mitigations to minimize the spread of antibiotic resistance in the environment.

MATERIALS AND METHODS

Description of study area

The study area is GWWTP and the downstream Notwane River receiving final effluent. Gaborone is the capital city of Botswana with a population of about 208,411 (Geonames database, 2018). GWWTP is situated along Notwane River, about 10 km North East from the main city center. The treatment plant has a capacity to receive 40,000 m³ waste per day; it receives water from households combined with industrial wastewater from Gaborone and surrounding areas. It is the biggest in the country consisting of the primary and secondary treatment stages which aims to degrade the biodegradable content of the sewage using the activated sludge processes. The process separates the solid material (sludge) from liquid (effluent). GWWTP does not have a tertiary treatment. Instead, the effluent wastewater is discharged into a series of maturation ponds for further biological degradation (Phuntsho et al., 2009). The final effluent from the maturation ponds is discharged into the Notwane River, which is the main source of water used by downstream farms for crop irrigation, mostly vegetables (Nkegbe et al., 2005). The water used for irrigation from this river is mostly effluent wastewater since there is normally no inflow from upstream especially during dry seasons.

Sample collection

Samples were collected from GWWTP; Site 1 (24°36.444'S; 25°57.811'E) influent wastewater (before treatment), Site 2 (24°36.575'S; 25°57.949'E) effluent (after treatment), and downstream of GWWTP along the Notwane River; Site 3 (24°35.348'S; 25°58.959'E) and Site 4 (24°33.808'S; 25°58.533'E). The distance between Site 2 and site 3 is 3.15 km and the distance between Site 3 and Site 4 is 5.16 km. Samples were collected once in a month all year round to cover all the four seasons; spring (September), summer (January), autumn (March) and winter (June). Three samples per site were taken using grab sampling method in the mornings between 8am and 10am. Samples were collected as

*Corresponding author. E-mail: rahubet@biust.ac.bw. Tel: +267 493 1530. Fax: +267 490 0102.

previously described by Yuan et al. (2015). Briefly 750 ml water samples were collected in 250 ml portions from three different points of the sites in sterile polystyrene bottles. Samples were placed in a cooler with ice-packs and transported to the laboratory and analyzed within 12 h.

Bacterial isolation, identification and antibiotic resistance characterization

Water samples were analyzed as previously described by Yuan et al. (2015) with few modifications. Briefly 0.1 ml aliquot of water samples from the ten-fold serial dilutions were spread plated on different selective agar media targeting different bacterial species that are of public health concern as shown in Table 1. The plates were incubated at appropriate temperature conditions (37 and 42°C) for 24 - 48 h using aerobic and anaerobic Incoterm economy Incubators (Labotec, South Africa). Isolates growing in respective selective media were randomly picked and confirmed by Gram stain procedure and biochemical tests (catalase, oxidase). An average of eight bacterial colonies in different media was then aseptically tooth-picked and further sub-cultured onto nutrient agar to obtain a pure culture. The pure culture isolates were grown in nutrient broth and subsequently stored in nutrient broth containing 50% glycerol (ratio, 1:1) at -80°C for further antibiotic resistance characterization.

Antibiotic resistance characterization

Antibiotic resistance analysis was performed from the stored isolates. Frozen isolates were thawed at room temperature and each bacterial isolate was aseptically sub-cultured into several 50 grid squared nutrient agar plates each containing different classes of clinically relevant antibiotics: ampicillin (32 µg/ml), cephalosporin (32 µg/ml), erythromycin (8 µg/ml), Streptomycin (64 µg/ml), sulfamethoxazole (512 µg/ml), meropenem (4 µg/ml), tetracycline (16 µg/ml), trimethoprim (64 µg/ml) ciprofloxacin (8 µg/ml), penicillin (16 µg/ml). Isolates were also sub-cultured on a nutrient agar without antibiotic as a control. The selected antibiotic concentrations used were defined as the Minimum Inhibition Concentration (MIC) of bacteria listed in Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS, 2015). These are universally accepted clinical breakpoint concentrations used in hospitals for treatment of bacterial infections. The plates were then incubated in appropriate aerobic/anaerobic and temperature conditions for 24 - 48 h. The resistance of isolates was recorded on the basis of growth in the presence of the corresponding antibiotic. Isolates resistant to more than two antibiotics were considered as multidrug resistant bacteria.

Statistical analysis of antibiotic resistant bacteria

To evaluate the associations on the frequencies (percentage occurrence) of antibiotic resistance phenotypes occurring in bacterial isolates from three categorical sources (influent, effluent and downstream environment,) a statistical analysis was conducted using Statistix 9.0 analytical software (<https://www.statistix.com/>). The Chi-square test was conducted with two degrees of freedom to generate the P values with significance levels of 0.01 and 0.05 considered.

DNA extraction and molecular characterization of antibiotic resistance

DNA from bacterial isolates (volume of 1 ml overnight broth

culture) was extracted as previously described by Mirmohammadsadeghi et al. (2013), with some modifications. Briefly, 1.5 ml overnight culture was centrifuged and the cell pellets were suspended in 600 µl of cell lysate solution (500 mM Tris-HCl, 200 mM EDTA, 460 mM NaOH; pH 8 and 15% SDS pH 6.6) and incubated at 80°C for 5 min. Then 3 µl of RNase was added and incubated at 37°C for 30 min. 200 µl of potassium acetate was added followed by 600 µl of isopropanol and the mixture centrifuged to pellet DNA. The DNA was then concentrated using ethanol, hydrated in 100 µl TE buffer and incubated overnight at room temperature.

The DNA was visually checked by gel electrophoresis (0.8% agarose gel (3 µl DNA plus 2 µl loading dye)). The quantity and quality of extracted DNA was determined using a nano drop spectrophotometer (Lasec, Jenway Genova nano) at an absorbance of 260 nm, and the DNA was stored at -20°C until polymerase chain reaction (PCR) amplification. ARGs were identified using qualitative PCR assay with primers specific for selected target genes as shown in Table 2. All PCR assays were performed in a 25 µl volume reaction consisting of 12.5 µl Emerald Amp[®] GT PCR Master Mix (TAKARA BIO INC), 1.5 µl each primer, 7.5 µl nuclease free water and 2 µl DNA template. Amplification was carried out in a PCR machine (ProFlex PCR system) using a temperature program consisting of initial denaturation of 95°C for 5 min, followed by 35 cycles of 98°C for 10 s, 1 min at the respective annealing temperature of various primers, 72°C for 1 min with a final extension at 72°C for 1 min. The annealing temperatures were calculated using a Tm calculator (Thermo Fisher Scientific Inc). Genomic DNAs of previously confirmed antibiotic resistant isolates were used as positive controls. PCR target positive products were analysed by gel electrophoresis using 1% (w/v) agarose stained in 4 µl/g ethidium bromide for 90 min in 1x TAE buffer and viewed under UV light (Gel doc-IT[®] imager UVP, Cambridge, UK). The sizes of the PCR products were confirmed against Quick-Load 1 Kb DNA ladder (BioLabs inc, England). The PCR primers were validated for correct target amplification by sub-cloning of the PCR products followed by Sanger sequencing.

RESULTS

Analysis of antibiotic resistant bacteria

A total of 565; 195 (influent), 216 (effluent) and 154 (downstream) isolates were characterized for antibiotic resistance. 526 (93.1%) isolates were found to be resistant to at least one antibiotic tested while only 39 (6.9%) were sensitive to all tested antibiotics. The percentage occurrence of resistant bacteria in the influent, effluent and downstream were 191 (97.5%), 204 (94.4%) and 131 (85.1%) respectively. The resistant bacteria occurring at the three sources were further characterized according to individual bacterial species. The isolates consisted of antibiotic resistant species; *Staphylococcus* (n=110), *Salmonella* (n=31), *Campylobacter* (n=37), *Listeria* (n=48), *Brucella* (n=38), *E. coli* (n=70), *Enterobacter aerogenes* (n=55) and *Pseudomonas* (n=137). The percentage occurrence of antibiotic resistance per individual species was expressed as percentage relative to the total number of isolates tested per sample source. For comparative purposes, bacterial species that had percentage resistance phenotypes equal or greater than 50% were

Table 1. Different selective media that were used in the study with their targeted bacterial species and the associated pathogenicity.

Media	Targeted bacterial species	Pathogenicity
Harlequin pseudomonas agar (LabM laboratories)	<i>Pseudomonas</i> species <i>Pseudomonas aeruginosa</i>	Eye, ear and skin infections
Harlequin salmonella TM ABC agar (LabM laboratories)	<i>Salmonella</i> species <i>Salmonella typhimurium</i>	Enteric fever, typhoid fever, food poisoning and gastroenteritis
Lab 112 campylobacter selective agar (LabM laboratories)	<i>Campylobacter</i> species <i>Campylobacter jejuni</i> <i>Campylobacter coli</i>	Inflammatory and Bloody diarrhea or dysentery.
Mannitol salt agar (Biolab laboratories)	<i>Staphylococcus</i> species <i>Staphylococcus aureus</i> <i>Staphylococcus epidermidis</i>	Skin infections, pneumonia and Bacteremia
Brucella agar (Conda laboratories)	<i>Brucella</i> species <i>Brucella abortus</i> , <i>Brucella melitensis</i>	Brucellosis-a zoonotic disease that may cause anemia, leucopenia and contagious abortion.
Chromo cult agar (Merk laboratories)	<i>Enterobacter aerogenes</i> <i>Escherichia coli</i>	Diarrhea, skin and soft tissue infections
Listeria selective agar (sigma-Aldrich)	<i>Listeria</i> species <i>Listeria monocytogenes</i>	Listeriosis-A food borne disease that affect newborn babies and may cause sepsis and meningitis.

Table 2. Primers used in the study.

Gene Target	Resistance phenotype	Primer sequence 5'-3'	Annealing temperature (°C)	Product size (bp)	References
<i>mphA</i>	Macrolides	F-GCCGATACCTCCCAACTGTA R-AGGCGATTCTTGAGCATTGC	52.8	403	Wang et al. (2017)
<i>sul1</i>	Sulfamethoxa- zole	F-GTGACGGTGTTCGGCATTCT R-TCCGAGAAGGTGATTGCGCT	54.7	921	Lanz et al. (2003)
<i>int1</i>	Class1 Integrase	F-GCATCCTCGGTTTTCTGG R-GGTGTGGCGGGCTTCGTG	50.4	457	Yousefi et al. (2010)
<i>dfp</i>	Trimethoprim	F-CCCAACCGAAAGTATGCGGTCCG R-GTATCTACTTGAT CGAT CAGG	45.6	171	Sunde (2005)
<i>strA</i>	Streptomycin	F-CCTGGTGATAACGGCAATTC R-CCAATCGCAGATAGAAGGC	50.0	548	Gebreyes et al. (2002)
<i>strB</i>	Streptomycin	F-ATCGTCAAGGGATTGAAACC R-GGATCGTAGAACATATTGGC	46.9	500	Gebreyes et al. (2002)
<i>tetA</i>	Tetracycline	F-CATATAATCATACCAATGGCA R-GGCGGTCTTCTTCATCATGC	46.2	500	Boerlin et al. (2005)
<i>tetB</i>	Tetracycline	F-CATTAATAGGCGCATCGCTG R-TGAAGGTCATCGATAGCAGG	50.5	93	Memon et al. (2016)
<i>ermA</i>	Erythromycin	F-GTTCAAGAACAATCAATACAGAG R-GGATCAGGAAAAGGACATTTTAC	45.2	421	Lina et al. (1999)
<i>ermB</i>	Erythromycin	F-AAAACTTACCCGCCATACCA R-TTTGGCGTGTTCATTGCTT	49.7	190	Zhai et al. (2016)

F; forward primer, R; reverse primer.

considered as high resistance while those less than 50% were considered low resistance. In addition, the Chi-square test was conducted and revealed there is statistical significance ($p < 0.05$) on the observed frequencies of antibiotic resistance phenotypes occurring in some bacterial species. Many did not yield a statistical difference ($p > 0.05$), results possibly affected by low sample sizes in the majority of bacterial species as shown in Table 3.

The *Staphylococcus* species from influent were highly

resistant against erythromycin (100%), cephalosporin (83%), trimethoprim (75%) and tetracycline (55%). Lower resistances occurred against sulfamethoxazole (43%), ampicillin (40%), penicillin (30%), streptomycin (8%) and meropenem (3%). Ciprofloxacin resistant *Staphylococcus* was not detected in the influent. The effluent species were highly resistant against cephalosporin (91%), erythromycin (71%) and penicillin (53%). Lower resistances were observed against trimethoprim (17%), tetracycline (49%), sulfamethoxazole (42%), ampicillin

Table 3. Occurrence and frequencies of antibiotic resistance phenotypes observed in bacterial species from influent, effluent and downstream.

Bacterial species	Source	^a High resistance(%)	^a Low resistance(%)	^b No resistance
<i>Staphylococcus</i> (n=110)	Influent (n=40)	*ERY (100), CEP (83), *TMP (75) TET (55)	SMX (43), AMP (40), PEN (30) STR (8)	CIP
	Effluent (n=53)	CEP (91), *ERY (71), PEN (53)	*TMP (17), TET (49), SMX (42) AMP (40), STR (8),MER (4),CIP (2)	
	Downstream (n=17)	PEN (64), TMP (65), *ERY (64) CEP (64), AMP (64), SMX (55)	TET (45),MER (9),STR (9)	MER, CIP
<i>Pseudomonas</i> (n=137)	Influent (n=46)	PEN (85),*TMP (85), *ERY (77) *CEP (69), AMP (54), *SMX (54)	*TET (38), CIP (15), STR (8)	MER
	Effluent (n=44)	*ERY (100), *CEP (100), AMP (80) PEN (80), *SMX (80),* TET (60)	*TMP (40), CIP (20), STR (10)	MER
	Downstream (n=47)	*ERY (100),* CEP (86), *TMP (79) AMP (71), PEN (64)	*SMX (43), *TET (21), CIP (7), STR (14) MER (14)	
<i>Salmonella</i> (n=31)	Influent (n=8)	ERY (100), TET (67), TMP (67) AMP (67)	PEN (33), CEP (33), SMX (33)	STR, MER CIP
	Effluent (n=10)	ERY (80), AMP (80), CEP (77) TET (60),PEN (60), TMP (60),SMX (60)	STR (10)	MER
	Downstream (n=13)	ERY (85), PEN (77), CEP (77) AMP (77), TMP (69), TET (54)	STR (23)	MER,CIP
<i>Campylobacter</i> (n=55)	Influent (n=18)	ERY (92), TRI (93), PEN (71) CEP (71), AMP (64), TET (50)	SMX (43),STR (29)	MER,CIP
	Effluent (n=21)	TET (95), PEN (95), SMX (95) AMP (86), TMP (81),CEP (81),ERY (71)	CIP (14), STR (10), MER (5)	
	Downstream (n=16)	TMP (88), AMP (69), SMX (56) ERY (56)	PEN (44), CEP (38), TET (31) CIP (38) STR (13), MER (6)	
<i>Listeria Species</i> (n=48)	Influent(n=16)	PEN (100), ERY (100), CEP (89) TMP (67)	TET (44), AMP (33), SMX (22)	STR, MER CIP
	Effluent (n=16)	PEN (88), ERY (63), CEP (75) TMP (50)	TET (38), AMP (44), SMX (44)	STR, MER CIP
	Downstream (n=16)	ERY (94), PEN (81), CEP (81) AMP (81), TMP (69), SMX (69)	TET (44), STR (6), MER (6)	CIP
<i>Brucella</i> (n=38)	Influent (n=13)	ERY (100), PEN (100), TMP (92), AMP (92),TET (77), SMX (77), CEP (69)	CIP (38) ,STR (8), MER (8)	
	Effluent (n=13)	AMP (100), ERY (92), TET (55) CEP (85), SMX (85), PEN (77)	STR (18), MEM (18), CIP (9)	CIP
	Downstream (n=12)	PEN (91), ERY (81), TMP (81) CEP (81),AMP (64),SMX (64),TET (55)	STR (18),MEM (18),CIP (9)	
<i>Enterobacter aerogenes</i> (n=55)	Influent (n=17)	ERY (94), TMP (88), AMP (82) PEN (82), SMX (82), CEP (59)	TET (47),CIP (12),STR (6)	MER
	Effluent (n=19)	PEN (89), CEP (79), ERY (74) TET (68),AMP (63),TMP (58), SMX (53)	STR (21), CIP (11)	MER
	Downstream (n=19)	ERY (95), PEN (79), AMP (79) CEP (79), SMX (74), TMP (74)	TET (47), CIP (11)	STR,MER
<i>Escherichia coli</i> (n=74)	Influent (n=26)	ERY (81), PEN (81), TMP (62) SMX (58), TET (54)	AMP (42), CIP (23), STR (23)	MER, CEP
	Effluent (n=28)	PEN (71), CEP (64), SMX (64) TET (57), AMP (54), ERY (54)	STR (14), TMP (46), CIP (7)	MER
	Downstream (n=20)	PEN (75), CEP (75), AMP (65), TET (60), TMP (60),ERY (60), SMX (55)	STR (25),CIP (15)	MER

^a; Resistance rates ≥ 50% were considered high resistance while those < 50% were considered low resistance, ^b; resistance phenotype not detected, statistical significance is highlighted in bold revealing Chi-square P values ; p < 0.05; * p <0.01; AMP; Ampicillin, CEP; Cephalosporin, CIP; Ciprofloxacin, ERY; Erythromycin, MER; Meropenem, PEN; Penicillin, SMX; Sulfamethoxazole, STR; Streptomycin, TET; Tetracycline, TMP; Trimethoprim

(40%), streptomycin (8%), meropenem (4%) and ciprofloxacin (2%). In the downstream, higher resistances occurred against penicillin (64%), trimethoprim (64%), cephalosporin (64%), ampicillin (64%), erythromycin (64%) and sulfamethoxazole (55%). Lower resistances were observed against tetracycline (45%), streptomycin (9%) and meropenem (9%). Ciprofloxacin resistant *Staphylococcus* species were not detected. The Chi-square test analysis on the *Staphylococcus* species revealed there is a statistically significant association among the sample sources for the following frequencies of resistance phenotypes; erythromycin ($P < 0.01$), cephalosporin ($p < 0.05$), trimethoprim ($p < 0.01$), penicillin ($p < 0.05$) but not for tetracycline, sulfamethoxazole, ampicillin and streptomycin as shown in Table 3.

A high resistance was observed against penicillin (85%), trimethoprim (85%), erythromycin (76%), cephalosporin (69%), ampicillin (54%) and sulfamethoxazole (54%) in *Pseudomonas* species from influent. Lower resistances occurred against tetracycline (38%), ciprofloxacin (15%) and streptomycin (7%). Meropenem resistant *Pseudomonas* species was not detected in influent. *Pseudomonas* species from effluent were also highly resistant against erythromycin (100%), cephalosporin (100%), ampicillin (80%), penicillin (80%), sulfamethoxazole (80%) and tetracycline (60%). Lower resistances were observed against trimethoprim (40%), ciprofloxacin (20%) and streptomycin (10%), while meropenem resistant *Pseudomonas* species were not detected in the effluent. Highly resistant *Pseudomonas* species occurred against erythromycin (100%), cephalosporin (86%), trimethoprim (79%), ampicillin (71%) and penicillin (64%) in the downstream environment, and lower resistances were observed against sulfamethoxazole (43%) tetracycline (21%), ciprofloxacin (7%), streptomycin (14%) and meropenem (14%). A statistically significant association was also observed for erythromycin ($p < 0.01$), cephalosporin ($p < 0.01$), trimethoprim ($p < 0.01$), penicillin ($p < 0.05$), tetracycline ($p < 0.01$), ampicillin ($p < 0.05$) and sulfamethoxazole ($p < 0.01$) with exception of only streptomycin as shown in Table 3.

Salmonella species from influent showed high resistances against erythromycin (100%), tetracycline (67%), trimethoprim (67%) and ampicillin (67%). Lower resistances were observed against penicillin (33%), cephalosporin (33%) and sulfamethoxazole (33%). The *Salmonella* species in the influent did not express resistance against streptomycin, meropenem and ciprofloxacin. In the effluent high resistances were detected against erythromycin (80%), ampicillin (80%), cephalosporin (70%), tetracycline (60%), penicillin (60%), trimethoprim (60%) and sulfamethoxazole (60%) while lower resistance occurred against streptomycin (10%). Meropenem resistant *Salmonella* species were not detected in the effluent. In the downstream environment, high resistances occurred against erythromycin (85%),

cephalosporin (77%), penicillin (77%), ampicillin (77%), sulfamethoxazole (69%), trimethoprim (69%) and tetracycline (54%). Lower resistances were observed against streptomycin (0.6%). Meropenem and ciprofloxacin resistant *Salmonella* species were not detected in the downstream environment.

The *Campylobacter* species in the influent were highly resistant against erythromycin (93%), trimethoprim (93%), penicillin (71%), cephalosporin (71%), ampicillin (64%) and tetracycline (50%). Lower resistances occurred against sulfamethoxazole (43%) and streptomycin (29%). Meropenem and ciprofloxacin resistant *Campylobacter* species were not detected in influent. Higher resistances were also observed from the effluent on *Campylobacter* bacteria against tetracycline (95%), penicillin (95%), sulfamethoxazole (95%), ampicillin (86%), trimethoprim (81%), cephalosporin (81%) and erythromycin (71%). The *Campylobacter* species showed lower resistances against ciprofloxacin (14%), streptomycin (10%) and meropenem (5%). The downstream environment *Campylobacter* species were highly resistant against trimethoprim (87%), ampicillin (69%), sulfamethoxazole (56%) and erythromycin (56%). Lower resistances occurred against penicillin (44%), cephalosporin (38%), tetracycline (31%), ciprofloxacin (19%), streptomycin (13%) and meropenem (6%).

Listeria species from influent showed higher resistances against penicillin (100%), erythromycin (100%), cephalosporin (89%) and trimethoprim (67%). Lower resistances were observed against tetracycline (44%), ampicillin (33%) and sulfamethoxazole (22%). Streptomycin, meropenem and ciprofloxacin resistant *Listeria* species were not detected in influent. The effluent *Listeria* species showed higher resistances against penicillin (88%), cephalosporin (75%), trimethoprim (50%) and erythromycin (63%). Lower resistances were observed against sulfamethoxazole (44%) and tetracycline (38%). Streptomycin, meropenem and ciprofloxacin resistant *Listeria* species were not detected in effluent.

The *Listeria* species occurring in the downstream environment had higher resistances against erythromycin (94%), penicillin (81%), cephalosporin (81%), ampicillin (81%), trimethoprim (69%) and sulfamethoxazole (69%). Lower resistances occurred against tetracycline (44%), streptomycin (6%), and meropenem (6%). Ciprofloxacin resistant *Listeria* species were not detected in downstream environment.

Brucella species from influent showed higher resistances against erythromycin (100%), penicillin (100%), trimethoprim (92%), ampicillin (92%), tetracycline (77%), sulfamethoxazole (77%) and cephalosporin (69%). Lower resistances occurred against ciprofloxacin (38%), streptomycin (8%) and meropenem (8%). In the effluent, *Brucella* species had a higher resistance against ampicillin (100%), erythromycin (92%), tetracycline (85%), cephalosporin (85%), sulfamethoxazole (85%) and penicillin (77%). Lower resistances were observed against

streptomycin (15%) and meropenem (8%).

In the downstream environment higher resistances were observed for penicillin (91%), trimethoprim (81%), cephalosporin (81%) erythromycin (81%), sulfamethoxazole (64%), and tetracycline at 55%. Lower resistances occurred with streptomycin (18%), meropenem (18%) and 9% of ciprofloxacin resistant *Brucella* species were detected.

E. aerogenes occurring in influent were frequently resistant against erythromycin (94%), trimethoprim (88%), ampicillin (82%), penicillin (82%), sulfamethoxazole (82%) and cephalosporin (59%). Lower resistances were observed against tetracycline (47%), ciprofloxacin (11%) and streptomycin (6%). Meropenem resistant *E. aerogenes* was not detected in the influent. The effluent *E. aerogenes* had a higher resistance against penicillin (89%), cephalosporin (79%), erythromycin (74%), tetracycline (68%), ampicillin (63%), trimethoprim (58%) and sulfamethoxazole (53%). Lower resistances occurred against streptomycin (21%) and ciprofloxacin (10%).

Meropenem resistant *E. aerogenes* bacteria were not detected. The *E. aerogenes* occurring in the downstream had a higher resistance against erythromycin (95%), penicillin (79%), ampicillin (79%), cephalosporin (79%), sulfamethoxazole (74%) and trimethoprim (74%). Lower resistances occurred against antibiotics tetracycline (47%) and ciprofloxacin (10%). streptomycin and meropenem resistant *E. aerogenes* were not detected in the downstream environment.

Lastly, *E. coli* from influent showed higher resistances against erythromycin (81%), penicillin (81%), trimethoprim (62%) sulfamethoxazole (57%) and tetracycline (54%), lower resistances were observed against ampicillin (42%), ciprofloxacin (23%) and streptomycin (23%). The GWWTP effluent bacteria showed higher resistances against penicillin (71%), cephalosporin (64%), sulfamethoxazole (64%), tetracycline (57%) ampicillin (54%) and erythromycin (54%). Lower resistances occurred against trimethoprim (46%), streptomycin (14%) and ciprofloxacin (7%). Meropenem resistant *E. coli* was not detected in GWWTP effluent.

E. coli from downstream environment showed a higher resistance against penicillin (75%), cephalosporin (75%), ampicillin (65%), tetracycline (60%), trimethoprim (60%), erythromycin (60%) and sulfamethoxazole (55%). Lower resistances occurred against streptomycin (25%) and ciprofloxacin (15%). Meropenem resistant *E. coli* bacteria were not detected in downstream environment.

Antibiotic resistance gene analysis

Diversity of ARGs was determined from DNA isolated in selected multiple resistant isolates. A total of 48 MDR isolates were selected for PCR analysis targeting ten ARGs. Six ARGs that showed positive amplification to the DNA samples tested were *tetA*, *mphA* and *dfr*, *sul1*, *int1*

and *strB*. Four targeted ARGs (*strA*, *ermA*, *ermB*, *tetB*.) were not detected in all 48 selected MDR bacterial species.

The frequency of detection of ARGs was expressed as percentage relative to the total number of selected isolates in each sample source. For comparative purposes, genes that were detected at 50% or more were considered most frequent than those below 50% as shown in Figure 1. ARGs that frequently occurred in influent were; *tetA* (75%), *mphA* (62.5%) and *dfr* (50%). Lower frequencies were detected for *sul1* (25%) and *int1* (25%). *strB* and *tetB* genes were not expressed in the influent. In effluent, *tetA* (62.5%) and *mphA* (50%) were frequently detected. *sul1* (37.5%), *dfr* (37.5%), *int1* (25%) and *strB* (12.5%) genes occurred at lower frequencies while *tetB* was not detected in the effluent. The downstream environment showed lower frequencies of all resistance genes targeted; *dfr* (37.5%), *mphA* (37.5%), *tetA* (25%), *sul1* (12.5%), *int1* (12.5%) and *strB* (12.5%). *tetB* was not detected.

Relative diversity of ARGs carried by individual bacterial species was also determined. *Campylobacter* species from influent were found to carry *tetA*, *dfr* and *mphA* resistance genes. *mphA* gene was the only one which was further detected in *Campylobacter* species from the effluent. There was no targeted gene detected from *Campylobacter* species in the downstream samples. In *Listeria* species, *tetA*, *dfr* and *mphA* genes occurred in influent. *int1* and *mphA* were also observed from *Listeria* species from effluent. The downstream species carried only *dfr* and *mphA* targeted genes. The *Brucella* species from influent carried *int1* and *mphA* genes. *tetA* occurred in the *Brucella* species from effluent and the downstream species showed presence of *int1*, *strB* and *mphA*.

Resistance genes that were observed in *E. coli* from influent were *tetA*, *int1*, *dfr*, *sul1* and *mphA*, effluent species had *tetA*, *sul1* and *mphA*. *tetA* and *mphA* further occurred in *E. coli* from downstream. *tetA*, *dfr* and *mphA* were observed in *E. aerogenes* bacteria from influent. *tetA*, *int1*, *strB*, *dfr* and *mphA* were detected in species from effluent.

In the downstream, only *dfr* gene was detected. *tetA* was the only gene that was detected in *Staphylococcus* species from influent and effluent. There was no other targeted gene that was detected in *Staphylococcus* species from the downstream environment. *tetA* and *sul1* occurred in *Pseudomonas* species from influent, effluent (together with *dfr*) and downstream environment as shown in Table 4.

DISCUSSION

Occurrence of potentially pathogenic and antibiotic resistant bacteria

The study detects different resistant and potentially

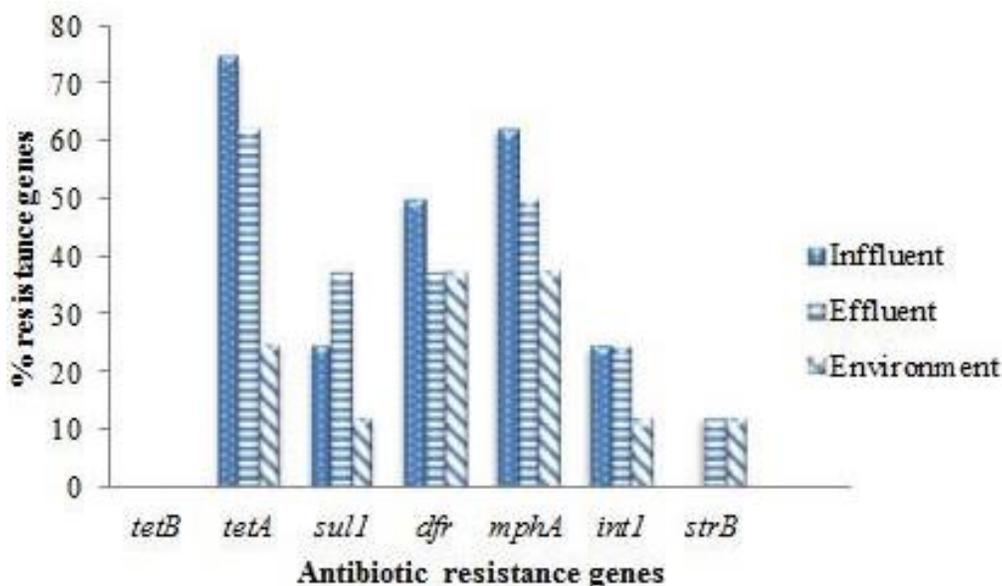


Figure 1. Occurrence of ARGs in the influent, effluent and downstream environment.

Table 4. ARGs present in bacterial species in the influent, effluent and downstream environment.

Bacterial species	Influent	Effluent	Environment
<i>Staphylococcus</i> species	<i>tetA</i>	<i>tetA</i>	*
<i>Pseudomonas</i> species	<i>tetA</i> , <i>sul1</i>	<i>tetA</i> , <i>sul1</i> , <i>dfr</i>	<i>tetA</i> , <i>sul1</i>
<i>Salmonella</i> species	*	<i>dfr</i> , <i>sul1</i>	<i>dfr</i>
<i>Campylobacter</i> species	<i>tetA</i> , <i>dfr</i> , <i>mphA</i>	<i>mphA</i>	*
<i>Listeria</i> species	<i>tetA</i> , <i>dfr</i> , <i>mphA</i>	<i>int1</i> , <i>mphA</i>	<i>dfr</i> , <i>mphA</i>
<i>Brucella</i> species	<i>int1</i> , <i>mphA</i>	<i>tetA</i>	<i>int1</i> , <i>strB</i> , <i>mphA</i>
<i>E. coli</i>	<i>tetA</i> , <i>int1</i> , <i>dfr</i> , <i>sul1</i> , <i>mphA</i>	<i>tetA</i> , <i>sul1</i> , <i>mphA</i>	<i>tetA</i> , <i>mphA</i>
<i>E. aerogenes</i>	<i>tetA</i> , <i>dfr</i> , <i>mphA</i>	<i>tetA</i> , <i>int1</i> , <i>strB</i> , <i>dfr</i> , <i>mphA</i>	<i>dfr</i>

*; no ARG gene target was detected

pathogenic bacterial strains; *Staphylococcus*, *E. coli*, *Brucella*, *Campylobacter*, *Listeria*, *Salmonella*, *Pseudomonas*, and *E. aerogenes* which were found to be resistant against clinically relevant classes of antibiotics (sulphonamides, tetracyclines, beta-lactams, macrolides, and aminoglycosides) in all the sample sources including effluent and downstream. High frequency of resistance was consistently observed for erythromycin, cephalosporins and trimethoprim across all bacterial species, with many of the isolates recording over 60% occurrence across all the samples sources (influent, effluent, and downstream). Considering the Chi-square test results, a statistically significant association was observed for only resistance phenotypes of *Staphylococcus* and *Pseudomonas* species in the influent, effluent and the downstream environment. The frequencies of antibiotic resistance phenotypes between *Staphylococcus* and *Pseudomonas* isolates vary considerably, with *Pseudomonas* species showing more

statistically significant associations to most tested antibiotics. Majority of ARB showed no statistical significance which could lead to conclusions suggesting no association in the frequencies of antibiotic resistance in the three samples sources. However, we cannot base our conclusions with the current dataset due to potentially low sample sizes in many instances. It is plausible nonetheless, to hypothesize no difference between the samples sources based on the efficiency or the design of the WWTP. Therefore, this warrants for further investigations in the future with increased sample size for more quantitative analysis of ARB occurring in three samples sources.

The presence of ARB in effluent wastewater and the downstream in the present study is supported by a study from Thomas and Nielsen (2005), which stated that ARB may still persist in the final effluent after treatment. Several studies have also demonstrated the spread of ARB from WWTPs to the downstream environments

(Figueira et al., 2011; Kümmerer, 2009; Da Silva et al., 2006). Multiple resistant bacteria such as *Staphylococcus aureus* (MRSA) was detected in Thibodaux sewage in USA (Boopathy, 2017; Naquin et al., 2014), and carried several resistance genes to beta-lactams antibiotics. All of the bacterial species detected are of clinical importance and may pose a serious concern to humans and livestock, contributing to the spread of uncontrollable infectious diseases such as Brucellosis caused by *Brucella* species (Corbel, 2006), Campylobacteriosis caused by *Campylobacter* species and Listeriosis by *Listeria* species (Robert, 2004). Tetracyclines, macrolides and cephalosporins are popular classes of antibiotics which include oxy-tetracycline, erythromycin and beta-lactam antibiotics respectively. The high frequency resistance detection in the isolates is not quite surprising. Resistance to trimethoprim was also among the most prevalent in bacterial species in all samples sources. Trimethoprim is also common, used for treatment of urinary tract infections and mostly used in combination with sulfamethoxazole for treatment of diarrhoea and Pneumocystis pneumonia in people with HIV/AIDS (Brolund et al., 2010; Eliopoulos and Huovinen, 2001). It is also critical to highlight the occurrence (although at very low frequency) of bacterial isolates resistant to meropenem (carbapenem) and ciprofloxacin (quinolone). All the bacterial species except *Salmonella* were found to be resistant to either meropenem, ciprofloxacin or both across all the sample sources. Carbapenems and quinolones are classes of antibiotics considered most effective against many multiple-drug resistant bacteria and has been listed by WHO as last resort antibiotics.

Diversity and dynamics of antibiotic resistance genes in different sources

A number of bacterial species were found to harbor more than one resistance gene associated with different antibiotic classes; tetracycline (*tetA*), sulphonamides (*sul1*), trimethoprim (*dfr*), macrolides (*mphA*), streptomycin (*strB*) and mobile gene cassettes (*int1*). The ARGs were also present in the bacterial species found in the effluent and downstream environment. ARGs *tetA*, *dfr* and *mphA* were most prevalent in the bacterial species across all sample sources. These results are consistent with some phenotypic characteristics discussed in the above paragraph, specifically for erythromycin and trimethoprim. Five targeted ARGs (*tetA*, *sul1*, *dfr*, *mphA* and *int1*) were observed in different bacterial species distributed across all sample sources. Bacteria are capable of accumulating multiple ARGs through integrative mobile elements (e.g. integrons), and can exchange genetic material through horizontal gene transfer facilitated by other mobile genetic elements such as plasmids (Rahube et al., 2014b). Therefore, ARGs can further be transferred to environmental bacteria and

ultimately to other bacteria that cause infections to humans and animals. The results are consistent with other studies conducted globally. For example, in a study by Ziemińska-Buczyńska et al. (2015) on communal WWTP in Zabrze (Poland), *dfr* and *sul1* genes were detected in most of the sample tested from influent, effluent and downstream. In another study, *sul1* together with *int1* were also detected in samples from WWTP and the downstream environments (Sayah et al., 2005). Diverse number of ARGs; *tetA*, *dfrA*, *catA*, *blaTEM*, *mphA* and *int1* were found in plasmids sequenced from Regina WWTP in Canada (Rahube et al., 2014). Other ARGs of clinical relevance; *aphA*, *aadA*, *oxa2*, *qacF*, *qnrC* and *qnrD* *tefB*, *tefE*, *tefW*, *tefM* and *tefZ*, were also detected in highly transferable plasmid groups and samples from WWTPs and the downstream environment in China and Germany (Xu et al., 2015; Schluter et al., 2007).

CONCLUSION AND RECOMMENDATIONS

Conclusively, GWWTP has the potential of disseminating clinically important bacterial pathogens including those that carry resistance genes to multiple antibiotics to the receiving environments. The effluent dominated Notwane River is an ideal hotspot for antibiotic resistance. Higher frequencies of potentially pathogenic ARB and ARGs to clinically relevant antibiotics in the effluent may suggest accumulation and proliferation of these antibiotic resistance determinants in maturation ponds before being released into the environment. Presence of antibiotic resistance determinants in the downstream still pose a threat to the public health if the bacteria can persist and proliferate, and further spread to food products especially vegetables irrigated with effluent-contaminated water. Furthermore, these antibiotic resistance determinants can spread to humans through ingestion of effluent contaminated vegetables, and also to livestock drinking from the river contaminated with effluent. The results from this study are critical in considering the development of new water quality monitoring schemes that also target antibiotic resistance determinants. We recommend to the Government health officials to carefully consider the environmental dissemination of antibiotic resistance. More extensive research on the environmental dimension of antibiotic resistance, targeting also vegetable farms and other end users of effluent contaminated water is necessary. Evidence from the research at national level is important for development and implementation of policies on the safe and sustainable use of effluent wastewater for irrigation purposes in Botswana and other developing countries.

CONFLICT OF INTERESTS

We declare that there is no conflict of interest regarding this study.

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