Occurrence of enterococci harbouring clinically important antibiotic resistance genes in the aquatic environment in Gauteng, South Africa

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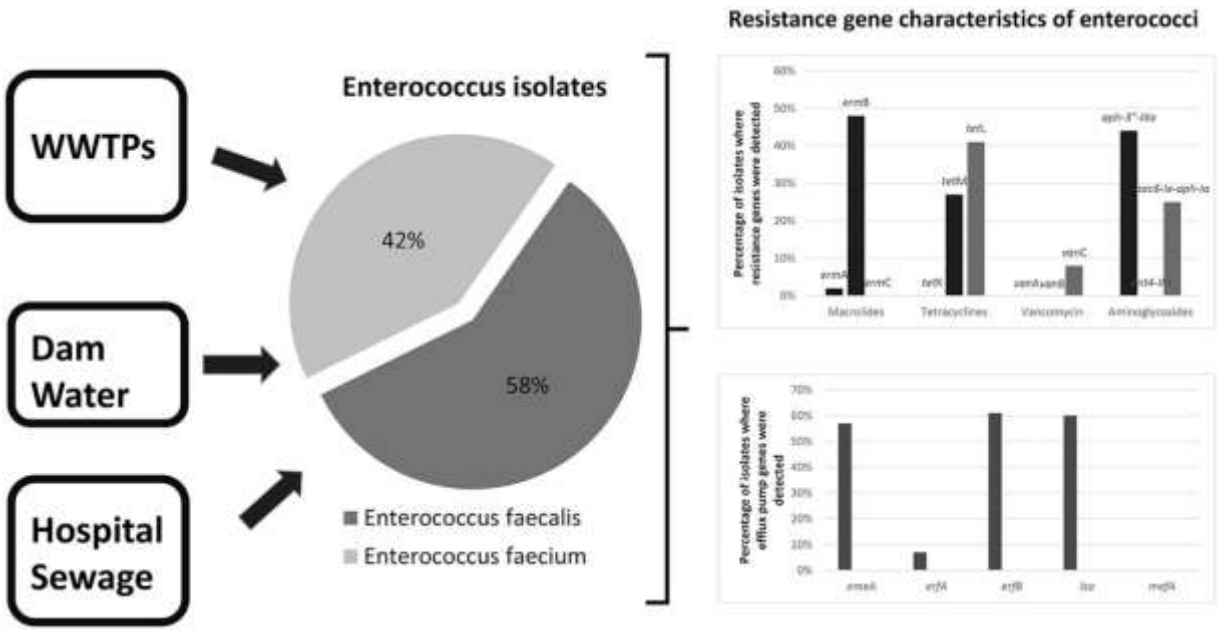
Highlights

- Various sewage wastewater and surface water samples were assayed for enterococci harbouring clinically relevant antibiotic resistance genes.
- *Enterococcus faecalis* was the most prevalent enterococci in the wastewater.
- *erm*B, *tet*M, *tet*L as well as *aph(3')-IIIa* resistance genes were frequently detected in the enterococci isolates.
- Wastewater treatment plants showed moderate removal of enterococci harbouring ARG.

Graphical abstract



Abstract

The development of antibiotic resistance and dissemination of its determinants is an emerging public health problem as it compromises treatment options of infections that were, until recently, treatable. Investigation of outbreaks of vancomycin resistant enterococci (VRE) suggests that the environment serves as a significant reservoir for antibiotic resistance genes (ARGs). However, there is a paucity of data regarding the presence of ARGs in the water sources in South Africa. In this study, water samples collected from wastewater treatment plants (WWTPs), surface water and hospital sewage were screened for enterococci harbouring genes conferring resistance to four classes of antibiotics. Enterococci isolates harbouring ARGs were detected in raw influent and treated wastewater discharge from WWTPs and hospital sewage water. Plasmid and transposon encoded *erm*B (macrolide), *tet*M and *tet*L (tetracycline) as well as *aph(3')-IIIa* (aminoglycosides) genes were frequently detected among the isolates, especially in *E. faecalis*. The presence of enterococci harbouring ARGs in the treated wastewater suggest that ARGs are discharged into the environment where their proliferation could be perpetuated. Among the enterococci clonal complexes (CCs) recovered from wastewater were *E. faecium* CC17 (ST18), which is frequently associated with hospital outbreaks and a novel *E. faecalis* sequence type (ST), ST780.

Capsule: Enterococci harbouring clinically relevant antibiotic resistance genes detected in South African water sources

Keywords: Wastewater, antibiotic resistance genes, enterococcus, water sources 44

1. Introduction

South Africa (SA) is a semi-arid, water-stressed country facing an ever-increasing demand for water. The Department of Water and Sanitation (DWS), formerly Department of Water Affairs and Forestry (DWAF), identified the reuse of huge quantities of treated wastewater discharged daily from wastewater treatment plants (WWTPs) into the aquatic environment as one of the strategies of augmenting the primary source of water (DWAF, 2004a). However, an estimated 80% of sewage water in some developing countries is discharged, without treatment, directly into drinking water sources (UN-Water, 2015a). Consequently, a variety of potentially infectious biological water contaminants, including enterococci, can be introduced into the water sources leading to potential adverse health risks to the public (Hamilton *et al*., 2007). Furthermore, WWTPs serve as a collection point for antimicrobial resistant bacteria and antibiotics disposed from households, hospitals, pharmaceutical factories and farmlands thereby creating an ideal environment for antibiotic resistance development and dissemination (Pruden *et al*., 2013). The discharge of bacteria harbouring antibiotic resistance determinants into water bodies such as dams or rivers may enhance the proliferation of antibiotic resistance in indigenous bacteria (Luo *et al*., 2014).

Enterococci are Gram-positive, non-spore forming bacteria that are ubiquitous in nature but found most abundantly in the gastrointestinal tract of animals and humans (Layton *et al*., 2010). The genus *Enterococcus* is composed of 40 different species of which, *Enterococcus faecalis* and *Enterococcus faecium* are the most commonly isolated species from human faeces and infections (Jackson *et al*., 2004). Although most enterococci are non-pathogenic, the two above-mentioned species have become increasingly important aetiological agents of hospital-acquired infections, especially in immunocompromised individuals (Fernandes *et al*., 2013). Beside causing illnesses such as bacteraemia, endocarditis, urinary tract infections as well as post-surgery wounds (Sydnor and Perl, 2011), enterococci have an intrinsic ability to resist a wide spectrum of antibiotics including

aminoglycosides and cephalosporins (Medeiros *et al*., 2014). Furthermore, the bacteria has a natural tendency to acquire and disseminate antibiotic resistance determinants against several antibiotics, including vancomycin, which is the treatment of choice for several serious Gram-positive bacterial infections (Tendolkar *et al*., 2003; Hayakawa *et al*., 2012). Antibiotic resistance can be ascribed to the acquisition of these genes through mobile genetic elements such as plasmids, transposons and through phages or mutations (Hegstad *et al*., 2012). Acquisition and dissemination of antibiotic resistance determinants can occur between different enterococci or from enterococci to other pathogenic bacteria such as, *Staphylococcus aureus* including methicillin-resistant *S. aureus* (Sievert *et al*., 2008). This could lead to a potentially significant threat to public health by limiting treatment options for *Enterococcus* species and other bacterial infections [De Niederhansern *et al*., 2004; World Health Organisation (WHO), 2014]. Enterococci may also possess efflux pumps (Levy, 2002) that mediate extrusion of a wide range of environmental chemicals, including antibiotics, thereby enabling them to persist in the environment.

Antibiotic resistant enterococci and antibiotic resistance genes (ARGs) have been detected in raw sewage and treated effluents of WWTPs (Auerbach *et al*., 2007; Zhang *et al*., 2009; Munir *et al*., 2011; Gao *et al*., 2012), hospital wastewater (Varela *et al*., 2013) and surface water (De Niederhäusern *et al*., 2013). In SA, despite their public health significance, very little is known about the presence of antibiotic resistant enterococci and ARGs in the aquatic environment. A study conducted in Alice, Eastern Cape reported the presence of ARGs in hospital wastewater and effluent of WWTPs (Iweriebor *et al*., 2015). However, the study only concentrated on the detection of vancomycin resistance genes. Data from vancomycin resistant enterococci (VRE) outbreaks that occurred in private and public hospitals in SA showed that the same strains were present in both the clinical and environmental samples, suggesting that the environment could serve as a reservoir for antibiotic resistant enterococci and resistance genes (von Gottberg *et al*., 2000; Mahabeer *et al*., 2016). The current study aims to investigate the presence of clinically important genes conferring resistance to several classes

of antibiotics including macrolides, tetracycline, glycopeptides and aminoglycosides in enterococci isolated from the water sources in Gauteng, SA. Clinically important genes are those genes that contribute significantly to bacterial unresponsiveness to antibiotics that are routinely used to treat clinical infections in SA and appears on the World Health Organization's list of antibiotics important for human medicine (WHO, 2016). The selected ARGs are important causes of antibiotic resistance and have been detected in clinical isolates of *E. faecalis and E. faecium* (Schmitz *et al.,* 2000; Hegstad *et al.,* 2010; Miller *et al.,* 2014). The study also screened the isolates for efflux pump and virulence genes that would enable them to persist in the environment and infect the human host. Knowledge of the presence of ARGs in the water sources is crucial in assessing their potential health risk to the public and mitigating their proliferation.

2. Experimental procedures

2.1. Study sites and sampling

Five WWTPs (WWTP1, WWTP2, WWTP3, WWTP4 and WWTP5) that discharge their treated effluents into the catchment of a downstream dam were selected for sampling. These WWTPs serve small towns located around the Vaal catchment area, SA, and receive domestic sewage, hospital and farmland wastewaters (Table 1 and Figure 1). From August 2015 to December 2015, wastewater samples (2 L), raw sewage water inflow (2 L) and treated wastewater discharge (2 L) were collected from each of the WWTPs on a monthly basis. An additional surface water sample (2 L) from a dam, downstream of the WWTPs where water is abstracted for purification and close to where the public use water for recreational activities was also sampled within the same period. Sewage water samples (2 L) were also collected from an academic hospital in Pretoria. Water samples were collected in sterile plastic containers and transported to the laboratory on ice. The samples were processed within 24 h after collection.

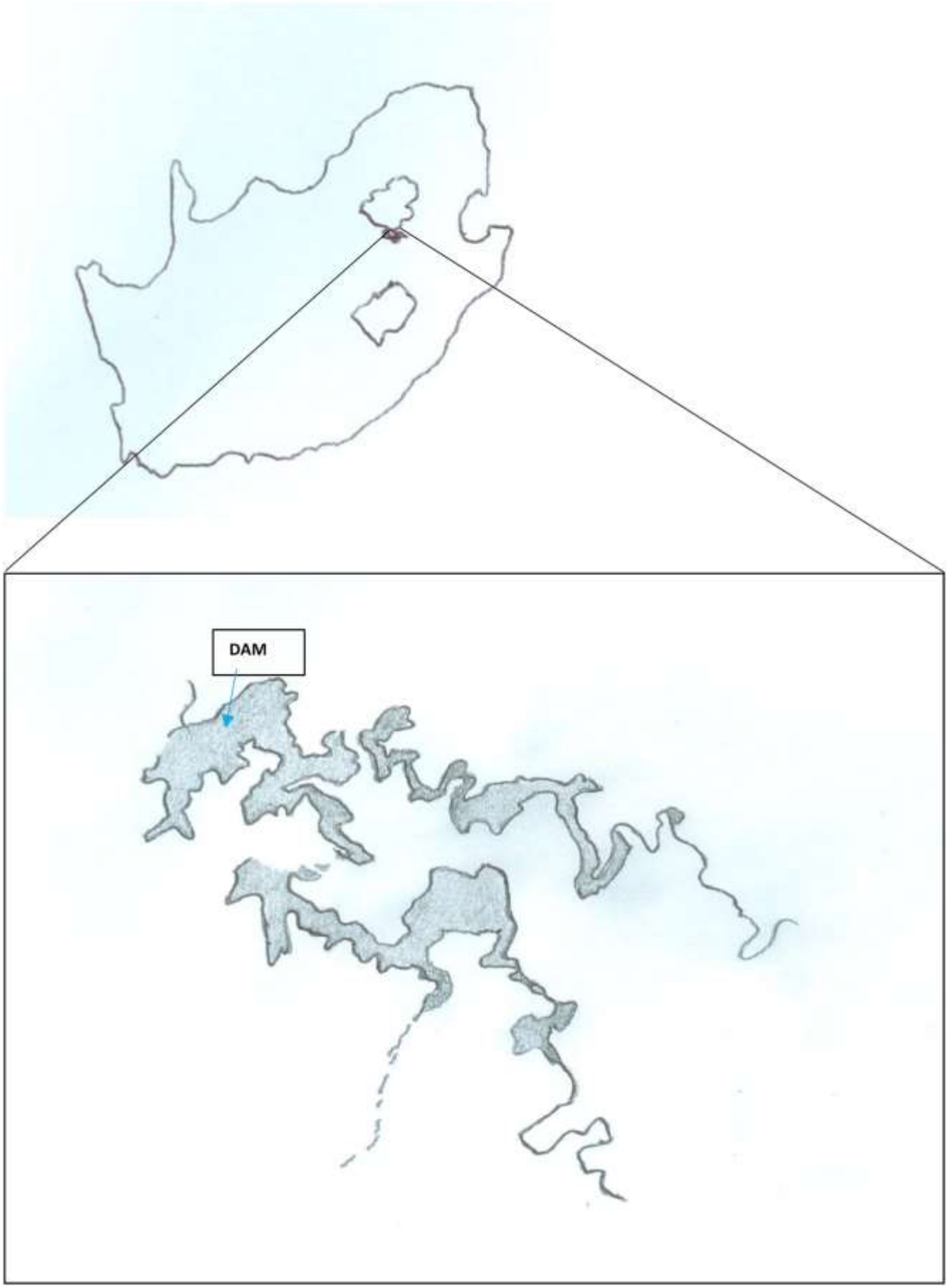


Figure 1: Map of South Africa showing the general location of the Vaal Dam catchment area

Table 1: Showing parameters associated with the functioning of the five WWTPs. 638

Wastewater treatment plant	Population (2011)	Economic activity	Main wastewater sources	Wastewater treatment technology	Operational capacity	Annual (2012) average effluent Quality compliance
WWTP1	19479	Tourism, farming	sewage	Biofilters and sludge beds	100% above capacity	58.2
WWTP2	5166	Tourism, sewage farming		Biofilters and sludge beds	100% above capacity	63.9%
WWTP3	701	Tourism, farming	Domestic sewage	Activate sludge, air	80% below capacity	66.4%
WWTP4	26144	Farming	sewage	Aerated and ponds	100% above capacity	Not available
WWTP5	17315	Tourism, Farming	sewage	Biofilters and ponds	100% above Capacity	Not available

2.2. Recovery of enterococci from water samples

Enterococci were recovered from the water samples by a standard membrane filtration procedure as described by the American Public Health Association (APHA, 1998). Briefly, 100 mL of each water sample was filtered through a nitrocellulose filter [0.45 µm pore size, 47 mm diameter (Fermentas, Waltham, MA)]. The filters were aseptically placed on chromogenic 51759 HiCrome™ Rapid Enterococci Agar media plates (Sigma-Aldrich®, St Louis, MO) and incubated at 37°C for 24 to 48 hours. Characteristic colonies of enterococci (blue for *E. faecalis* and green for *E. faecium*) were sub-cultured onto blood agar plates and resulting colonies were Gram-stained. Presumptive colonies of each of the *E. faecalis* and *E. faecium* isolates were inoculated into 5 mL of 3.7% brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) and incubated for 24 hours at 37°C. The broth cultures were stored at -70°C in 50% sterile glycerol solution until further analysis.

2.3. Identification of Enterococcus species

The identity of the presumptive enterococci isolates were confirmed by a multiplex PCR assay as described by Depardieu *et al.* (2004) using *E. faecalis* and *E. faecium* species specific primers targeting the *ddl* (D-Ala–D-Ala ligase) gene (Table 5, supplementary material). Briefly, genomic DNA was extracted from 2 mL of presumptive enterococci isolates grown overnight in BHI using the ZR Fungal/Bacterial DNA Miniprep™ commercial kit (Fermentas, Waltham, MA) according to the

manufacturer's instructions. The 25 µL multiplex PCR reaction consisted of 2X PCR mastermix (Bioline®, UK), 0.2 µM for each primer and <1 µg DNA. The PCR amplification was performed in a G-storm thermocycler (Vacutec, UK) using the following conditions: an initial denaturation step of 95°C for 5 min, followed by 30 cycles of denaturing at 95°C for 30 s, annealing at 58°C for 30 s, and extension step at 72°C for 1.5 min, with a final extension at 68°C for 1 min. Genomic DNA from *E. faecalis* ATCC 51299 and *E. faecium* ATCC 51559 were used as positive controls and sterile water as negative control. PCR products were visualised under UV light (TFM-26 Ultra Transilluminator, Upland, CA) after gel electrophoresis [SeaKem® LE agarose (1.5%), Lonza, Allendale, NJ) and staining in ethidium bromide [(10 µg/mL) Promega Corp., Madison,WI].

2.4. Detection of antibiotic resistance, efflux pump and virulence genes

Genes conferring antibiotic resistance to macrolides (*erm*A, *erm*B and *erm*C), tetracycline (*tet*K, *tet*M and *tet*L), vancomycin (*van*A, *van*B and *van*C) and aminoglycosides *aph(3')-IIIa, ant(4')-Ia, aac(6')-Ie-aph(2")-Ia*) as well as the presence of efflux pump (*erf*A, *erf*B, *eme*A and *lsa*) and virulence genes (*asa*, *esp*) were screened by multiplex PCR using published primers (Stoll *et al*., 2012; Kwon *et al*., 2012; Duran *et al*., 2012; Kang *et al*., 2013) (Table 4, supplementary material). The 25 µL multiplex PCR reaction consisted of 2X PCR mastermix (Bioline®, UK), 0.2 µM for each primer and <1 µg DNA. The PCR conditions consisted of an initial denaturation step of 95°C for 5 min, followed by 30 cycles of denaturing at 95°C for 30 s, annealing at 57°C for 1 min, and extension step at 72°C for 1 min, with a final extension at 72°C for 8 min. Genomic DNA from *E. faecalis* ATCC 51299 and *E. faecium* ATCC 51559 were used as positive controls and sterile nuclease free water as negative control. A similar PCR reaction mixture was used to screen for the presence of aggregation substance factor (*asa*) and extracellular surface protein (*esp*) in *E. faecalis* and *E. faecium* isolates using primers described by Comerlato *et al.,* (2013) (Table 5, supplementary material). The PCR conditions involved an initial denaturation step of 95°C for 2 min, followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min.

2.5. Molecular typing of enterococci isolates

Enterococci isolates were typed using the PFGE technique as described by McDougal *et al.,* (2003). The chromosomal DNA of the enterococci isolates was digested by SmaI and ApaI (New England Biolabs, Ipswich, MA) as primary and secondary restriction enzymes respectively. The resulting banding patterns were analysed using the GelCompar II software (Applied Maths, Belgium). The PFGE banding patterns of the isolates was clustered using the Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) in order to construct dendrograms. Pulsotypes were defined as isolates sharing ≥80%. Major pulsotypes included five or more isolates, while minor pulsotypes included less than five isolates.

Three randomly selected isolates from each of the two species were typed by MLST following a scheme based on the internal sequences of seven housekeeping loci (Table 2) and using primers and conditions described by Ruiz-Garbajosa *et al.,* (2006) for *E. faecalis* and Homan *et al.,* (2002) for *E. faecium* (Table 5, supplementary material). Briefly, the PCR assay was done in a 25 µL volume consisting of 2X Mastermix (Bioline®, UK), 0.5 µM each primer and <1 µg DNA. The PCR conditions consisted of an initial denaturation at 95°C for 15 min; 32 cycles at 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min, and a final extension step at 72°C for 7 min. The PCR products were detected by gel electrophoresis and purified using a PCR purification kit (Qiagen, Hilden, Germany). The purified PCR amplicons were sequenced using forward and reverse primers and the resulting sequences were analysed using CLC Bio workbench version 6.0 (Qiagen). The sequences were uploaded in the pubMLST database (http://pubmlst. org/efaecium/ and http://pubmlst.org/efaecalis/) for analysis and comparisons. The combination of the seven obtained alleles for each isolate was assigned a specific sequence type (ST) and the clonal complex (CC) was determined using eBURST

Table 2: Distribution of *E. faecalis* and *E. faecium* in water samples collected from WWTPs, 664 downstream dam and hospital wastewater.

	WWTPs			
Bacterium	**Sewage Water**	**Treated Effluent**	**Hospital**	**Dam**
E. faecalis	39	8	15	0
E. faecium	17	11	17	0

3. Results

3.1. Isolation and detection of enterococci from water samples

Forty-eight (48) water samples, which included raw sewage inflow (20), treated wastewater discharge (20), downstream surface water (4) and hospital wastewater (4) were collected over a period of five months (August to December 2015) and analysed for *E. faecalis* and *E. faecium* by both culture and multiplex PCR assays. One hundred and seven (107) enterococci were isolated from the water samples, of which 58% (62/107) were identified as *E. faecalis* and the remaining 42% (45/107) as *E. faecium* (Table 2). The majority (52%, 56/107) of enterococci were detected from the raw sewage water, followed by hospital wastewater (30%, 32/107) and the treated wastewater discharge (18%, 19/107). Neither *E. faecalis* nor *E. faecium* were detected in the surface dam water downstream of the WWTPs. Furthermore, the number of *E. faecalis* (70%, 39/56) present in raw sewage water samples exceeded that of *E. faecium* (30%, 17/56).

Across the five WWTPs, 33% (13/40) of both WWTP2 and WWTP3 sewage water samples were positive for *E. faecalis and E. faecium* followed by WWTP1 (25%, 10/40); and 18% (7/40) for both WWTP4 and WWTP5 (Figure 1). The two *Enterococcus* species were detected less frequently in the treated effluents, ranging from 0% in WWTP3 and WWTP5 to 33% (13/40) in WWTP4, indicating a varied efficiency in bacterial removal by the treatment plants, with WWTP3 and WWTP5 appearing to be the most efficient in removing enterococci from the wastewater and WWTP4 the least efficient.

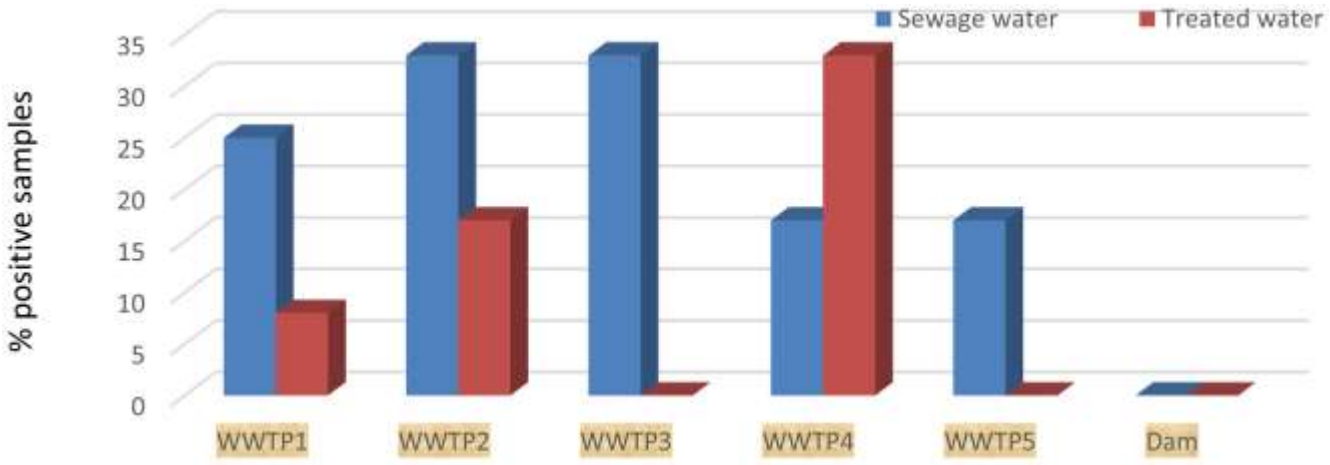


Figure 2: Prevelance of *E. faecalis* and *E. faecium* in sewage and treated wastewater effluent samples in all five WWTPs

3.2. Detection of antibiotic resistance, efflux pump and virulence genes

3.2.1. Antibiotic resistance genes

The *E. faecalis* and *E. faecium* isolates were screened for the presence of genes conferring resistance against four selected antibiotic classes using multiplex PCR assays. As shown in Table 3, the *erm*B gene was the only macrolide resistance gene detected and was present in 45% (48/107) of the enterococci isolates (26/107 in *E. faecalis*, 22/107 in *E. faecium* isolates). Tetracycline resistance genes (*tet*M and *tet*L) were detected in 44% (47/107) and 41% (44/107) of all enterococci isolates respectively. Between the two species, the *tet*M gene was present in 45% (28/62) of *E. faecalis* and 42% (19/45) of *E. faecium* isolates, whereas the *tet*L gene was detected in 29% (18/62) of *E. faecalis* and 58% (26/45) of *E. faecium* isolates. The genes conferring resistance to the aminoglycosides, the *aph(3')-IIIa* was the most prevalent in all enterococci (44%, 47/107) followed by the *aac(6')-Ie-aph(2")-Ia* (25%, 27/107) gene. Between the two species, the *aph(3')-IIIa* and *aac(6')-Ie-aph(2")-Ia* genes were more prevalent in *E. faecalis* (34% and 25%, respectively) as compared to *E. faecium* (10% and 14% respectively). The vancomycin resistance gene, *van*C was found in 8% (9/107) of all enterococci isolates, (6/107) in *E. faecalis* and (3/107) in *E. faecium*. The two resistant genes that contribute greatly to VRE, the *van*A

and *van*B, were not detected in the isolates, indicating that VRE were not prevalent in the sampled water sources at the time. Overall, the majority 80% (86/107) of enterococci isolates harboured resistance genes to two or more classes of antibiotics.

Table 3: Detection of genes conferring resistance to macrolides, tetracycline, vancomycin and 700 aminoglycosides as well as efflux pump and virulence genes in enterococci isolates

		Number of positive isolates		
Antibiotic	**Resistance gene**	***E. faecalis***	***E. faecium***	**Total**
Macrolides	*erm*A	-	-	-
	*erm*B	26	22	48
	*erm*C	-	-	-
Tetracycline	*tet*K	-	-	-
	*tet*M	28	19	47
	*tet*L	18	26	44
Vancomycin	*van*A	-	-	-
	*van*B	-	-	-
	*van*C	6	3	9
Aminoglycosides	*aph(3')-IIIa*	36	11	47
	ant(4')-Ia	-	-	-
	aac(6')-Ie-aph(2")-Ia	15	12	27
Efflux pump	*erf*A	3	4	7
	*erf*B	56	8	64
	*eme*A	53	7	60
	lsa	54	9	63
	*mef*A	-	-	-
Virulence genes	*asa*	35	16	51
	Esp	32	12	44

Across the five WWTPs, ARGs were more prevalent in raw sewage water (75%, 56/75) compared to the treated discharge (25%, 19/75), suggesting that the treatment plants were capable of removing the enterococci harbouring ARGs but not efficient enough for total elimination. The WWTP1, WWTP3 and WWTP5 were efficient in bacterial removal as shown by reduction in the number of *E. faecalis* and *E. faecium* harbouring resistance genes. Conversely, the number of enterococci harbouring ARGs in the treated effluent of WWTP2 and WWTP4 was similar to those in the raw sewage water. This

indicated that these two WWTPs are ineffective in removing the enterococci and consequently, the bacteria carrying the resistance genes could be discharged into water sources used for domestic, irrigation and recreational purposes as effluent. Not surprisingly, the highest number of enterococci harbouring ARGs was found in the hospital wastewater (Table 3) since this is where most enterococcus infections and antibiotic treatment takes place.

Table 4: Distribution of antibiotic resistance genes in the raw sewage and treated effluent water 735 samples across the five WWTPs and hospital sewage water

	Number of enterococci harbouring antibiotic resistance genes					
Water Sample	**WWTP1**	**WWTP2**	**WWTP3**	**WWTP4**	**WWTP5**	**Hospital**
Raw sewage	8	8	18	7	15	32
Treated effluent	1	9	-	9	-	N/A
Total	9	17	18	16	15	32

3.2.2. Efflux pumps and virulence genes

Enterococci isolates were also screened for efflux pump genes by multiplex PCR assays and the results are summarised in Table 3. In general, efflux pump genes *erf*B, *eme*A and *lsa* were more prevalent [60%, (64/107), 56% (60/107), 59% (63/107) respectively] among enterococci isolates than *erf*A (7%, 7/107). Between the two species, the *erf*B, *eme*A and *lsa* genes were more prevalent in *E. faecalis* [52% (56/107), 50% (53/107), 50% (54/107) respectively] than in *E. faecium* [7%, (8/107), 7% (7/107), 8% (9/107) respectively]. The *mef*A gene was not detected in any of the enterococcal isolates from this study. Two virulence genes, aggregation substance factor (*asa*) and extracellular surface protein (*esp*) were also screened in all enterococci isolates and as shown in Table 3, the two genes were present in 48% (51/107) and 41% (44/107) of the isolates respectively. The *E. faecalis* isolates tested positive for more virulence genes than *E. faecium*.

3.2.3. Molecular typing of E. faecalis and E. faecium isolates

Thirty-eight percent (39%, 24/62) of *E. faecalis* and 96% (43/45) of *E. faecium* isolates were successfully digested with SmaI endonuclease and their fragment profiles determined by pulsed field gel electrophoresis (PFGE). A dendrogram of percent similarity, calculated with Dice coefficient from PFGE data using a cut off of ≥80% revealed a high genetic diversity of the isolates (Figure 3a and 3b). The PFGE analysis of the *E. faecalis* isolates showed only one major pulsotype cluster, which comprised of six isolates recovered from hospital wastewater and had more than 80% similarity. The PFGE analysis of the *E. faecium* isolates revealed a high genetic diversity. Four percent (2/45) of the isolates clustered together to form a minor pulsotype with a similarity value of ≥ 80% (Fig. 3b). Both isolates were recovered from treated effluents of the same WWTP. An attempt to redigest isolates, not digested by SmaI, with a secondary restriction enzyme ApaI was also unsuccessful and were, therefore, classified as untypable.

Three random isolates from each of the two enterococcus species were selected for multilocus sequence typing (MLST) analysis and were assigned sequence type numbers: ST23 (CC23), ST25 (CC25) and ST780 for *E. faecalis* isolates, and ST18 (CC17) and ST32 (CC22) for *E. faecium* isolates. Isolates assigned ST23 and ST25 were recovered from hospital wastewater and influent sewage water, respectively. The isolate assigned ST780 had never been reported before and was assigned a ST after being sent to the curator. The isolate was given the allelic profile (95-8-12-93-40-15-91) and assigned ST780 as a novel sequence type. Two *E. faecium* isolates assigned ST32 were obtained from the sewage water and treated discharge from WWTP and shared the same allelic profile: 3-3-1-2-1-1-1.

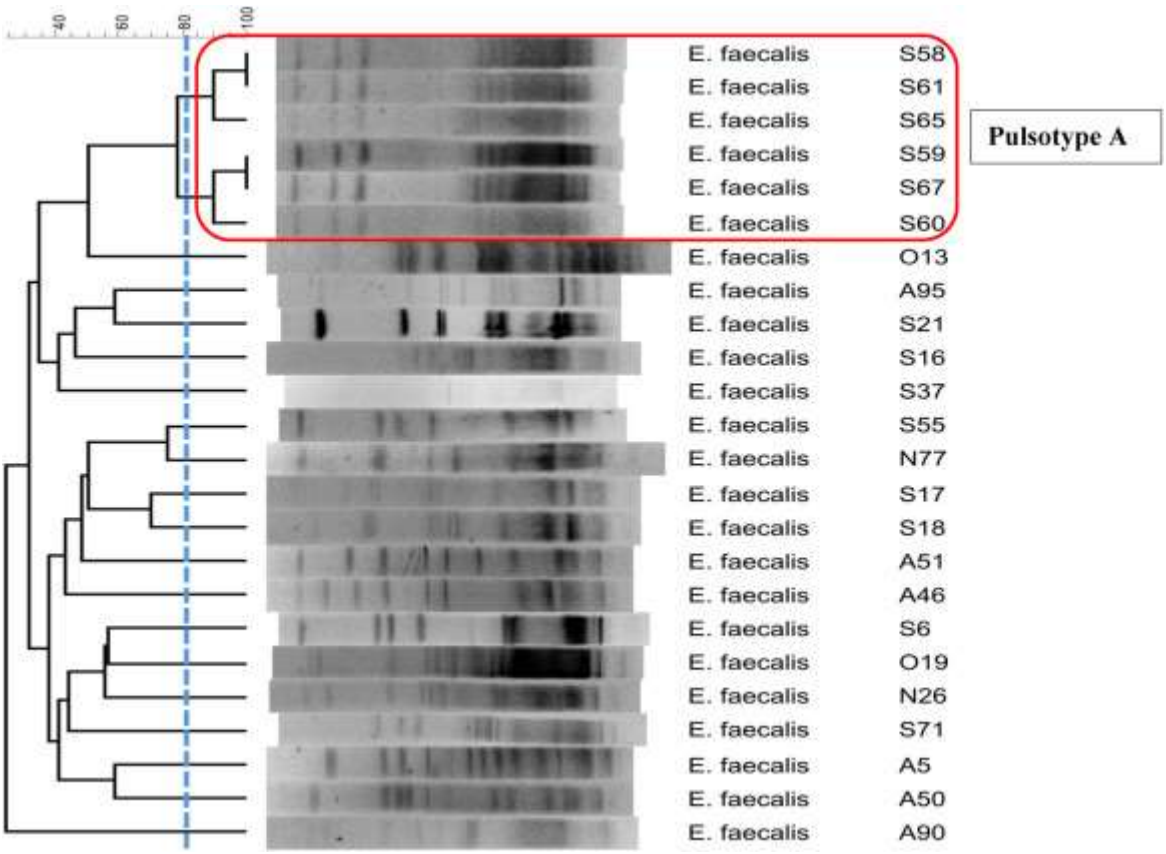


Fig. 3a. Dendrogram of PFGE analysis showing the high genetic variation among the *E. faecalis* isolates.

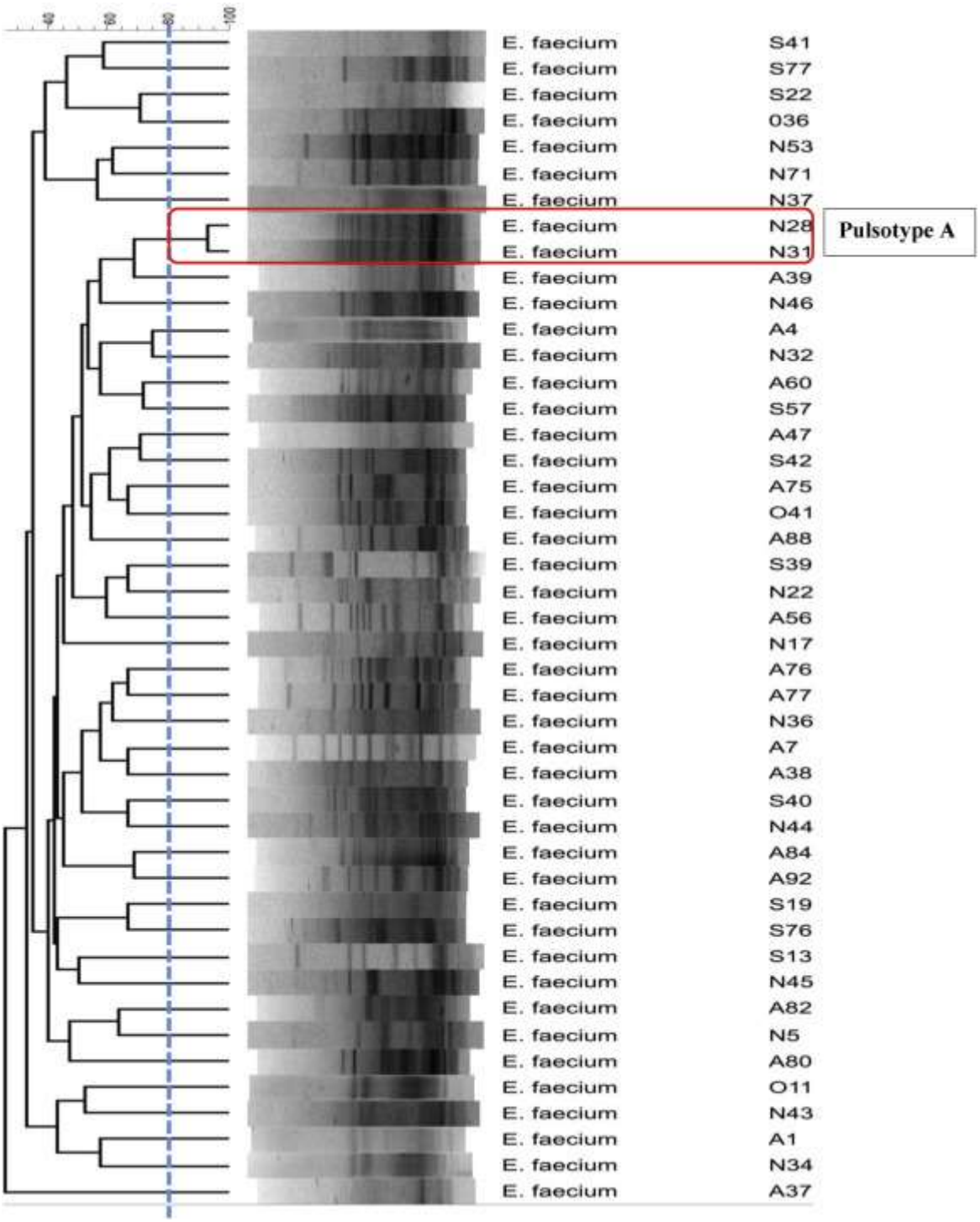


Fig. 3b. Dendrogram of PFGE analysis showing the high genetic variation among the *E. faecium* isolates.

4. Discussion

Antibiotic resistance is increasingly compromising treatment options for pathogenic bacteria that were, previously, susceptible to a variety of antimicrobial compounds. The WHO declared development and dissemination of antibiotic resistance as one of the major public health threats of

the 21st century (WHO, 2014). Investigation into outbreaks that occurred in private and public hospitals in SA showed that the similar strains were present in both the clinical and environmental samples (Mahabeer *et al*., 2016), suggesting that the environment could serve as an important reservoir for antibiotic resistant enterococci and resistance genes. Yet, there is a paucity of data on the presence of ARGs in the aquatic environment, thereby limiting our ability to assess their potential health risk to the public and mitigate their proliferation. Therefore, the present study was conducted to screen for clinically important genes conferring resistance to four classes of antibiotics in enterococci isolated from WWTPs, surface water, as well as, hospital wastewater so that their presence could prompt enhanced removal and mitigate their propagation in the aquatic environment.

The present study observed that *E. faecalis* was more prevalent in the wastewater samples as compared to *E. faecium*. This is not surprising as municipal wastewaters are the conduit of faecal material from humans and animal farmlands in which *E. faecalis* is the most dominant species relative to *E. faecium*. This observation is supported by results from a similar study in Alice, Eastern Cape, which detected more *E. faecalis* in municipal wastewater than *E. faecium* (Iweriebor *et al*., 2015). However, prevalence of the two *Enterococcus* species in wastewater varies between countries. *Enterococcus faecalis* was found to be the most prevalent enterococci both in municipal and hospital sewage waters in Sweden, whereas *E. faecium* was predominant in similar sample types in the United Kingdom (UK) and Spain (Kuhn *et al*., 2003). In Tunisia, *E. faecium* was reported to be the most prevalent enterococci between the two species (Ben Said *et al*., 2015).

Vancomycin is used to treat serious nosocomial bacterial infections that have become non-responsive to first line antibiotics. Resistance to vancomycin is mediated by proteins encoded by the *van*A, *van*B and *van*C genes, with the first two being the most clinically significant as they confer higher-level resistance than *van*C. The present study could only detect *van*C in 8% of both *E. faecalis* and *E. faecium* isolates, suggesting that vancomycin resistance is not widespread. However, this observation is in

contrast to findings by Iweriebor *et al* (2015) who reported that over 80% enterococci isolates from WWTPs and hospital wastewater in Alice, Eastern Cape, SA harboured *van*B and *van*C genes. The largely rural Eastern Cape town of Alice has many commercial pig farms, where antibiotics are used in the management of farm animals. The use of antibiotics, such as avoparcin, has been associated with high level of VRE in farm animals (Boerlin *et al*., 2001) and as a collection point, all WWTPs close to these farms are likely to contain higher numbers of these bacteria

The antibiotic resistance genes, *erm*B (macrolide), *tet*M and *tet*L (tetracycline) as well as *aph(3')-IIIa* (aminoglycosides), which are also clinically important (routinely used to treat clinical infections caused by Gram positive bacteria) were common among the enterococci isolates. Most of these genes are located on mobile genetic elements such as plasmids and conjugative transposons (Simjee and Gill, 1997; Valrado *et al*., 2009), as such they are easily spread within the genus or genera. The presence of ARGs in WWTPs effluent is concerning as the water bodies receiving the effluent downstream such as, rivers and dams could be polluted and result in further propagation. In this study, no antibiotic resistant bacteria were detected in a water sample from the downstream dam, possibly due to being diluted out. However, several studies have reported the presence of antibiotic resistant bacteria in surface waters (Schwartz *et al*., 2002).

The presence of enterococci and ARGs in the treated effluent indicates that some WWTPs investigated in the study were not efficient in removing the biological contaminants in the wastewater. Different WWTPs use different treatment technologies, including aerated and facultative ponds, activated sludge and diffuse air, biofilters and sludge oxidative ponds that are said to have uniform relative efficiency (Sala-Garrido *et al*., 2011). However, due to the increase in population and state of disrepair, WWTPs operate beyond their designed capacity (Edokpayi *et al*., 2015), leading to the discharge of inadequately treated wastewater containing bacteria harbouring ARGs. In this study, only WWTP3 operated below its designed capacity, while the other treatment plants (including WWTP4, which

showed the least bacteria removal) operated almost 100% over the designed capacity and therefore did not meet good practice and regulatory expectations (DWAF, 2013). The presence of enterococci and ARGs in the treated discharge may also indicate the intrinsic ability of enterococci to resist environmental stress (Renner and Peters, 1999). While significant in enterococci, their presence is not an indication of phenotypic expression of antibiotic resistance. It would therefore be recommended that future studies perform antibiotic susceptibility testing on study isolates to determine phenotypic expression of antibiotic resistance.

Enterococci possess efflux pumps that mediate extrusion of a wide variety of structurally unrelated environmental chemicals and antibiotics (Saier *et al*., 1999; Levy, 1992), thereby enabling them to persist in the environment. In the present study, efflux genes *erf*B, *eme*A and *lsa* were more prevalent in the isolates, especially *E. faecalis* isolates and could explain why a relatively higher number of *E. faecalis* were detected in the treated effluent. Virulence markers are found in enterococci possibly due to conjugation that occur in the environment or the gastrointestinal tract of animals and humans (Sabia *et al*., 2008; Eaton and Gasson, 2001). In this study, the *asa* gene, which encodes for the aggregation substance that mediates efficient contact and facilitates plasmid exchange of ARGs between donor and recipient bacteria, and the *esp* gene, which is associated with colonisation of urinary tract epithelial cells and biofilm formation, were more prevalent in *E. faecalis* as compared to *E. faecium*, an observation supported by reports from other studies (Pangallo *et al*., 2004; Pangallo *et al*., 2008; Hallgren *et al*., 2009; De Niederhäusern *et al*., 2013).

There was a high genetic diversity among the *E. faecalis* and *E. faecium* isolates as indicated on the PFGE dendrogram. This is not surprising as these WWTPs receives enterococci from a wide variety of sources including, domestic sewage, agricultural and hospital wastewater, and multiple studies have reported similar findings (Castillo-Rojas *et al.*, 2013; Nam *et al*., 2013; Ben Said *et al.*, 2015). Except for the novel ST780 reported in the present study, the other two *E. faecalis* sequence types ST23 and

ST25, recovered from hospital wastewater and WWTP, respectively, belong to clonal complex (CC) 23 and CC25 that are commonly isolated from a variety of sources including animals, aquatic environments, food and hospitalised patients (Quiñones *et al*., 2009). The *E. faecium* ST18 (from hospital sewage) belongs to CC17, a widely disseminated CC that has been isolated in many countries and has been associated with hospital outbreaks (Freitas *et al*., 2009; Lasch *et al.*, 2014). The two *E. faecium* isolates assigned ST32 were recovered from the influent raw sewage and treated discharge of the same WWTP (WWTP4), indicating that these strains were resistant to sewage treatment.

Sampling in the present exploratory study was done for a short period (August to December 2015) and as such seasonal abundance patterns of enterococci and ARGs could not be established. A further longitudinal study is therefore required to have a complete picture of seasonal distribution patterns. Other studies have, reported differences in seasonal distribution of ARGs in Germany with autumn and winter having a higher abundance of ARGs (Caucci *et al*., 2016).

5. Conclusion

The present study has shown that the five WWTPs and hospital wastewater contained enterococci carrying clinically relevant genes conferring resistance to four classes of antibiotics. Plasmid and transposon encoded *erm*B (macrolide), *tet*M and *tet*L (tetracycline) as well as *aph(3')-IIIa* (aminoglycosides) genes were common among the enterococci isolates, especially in *E. faecalis* isolates. The *van*A and *van*B gene, that contribute greatly to vancomycin resistance were not present in the isolates. Some of the WWTPs were not efficient in removing ARGs from wastewater as shown by their presence in the treated wastewater discharge. Rivers and dams, therefore, could be polluted with ARGs, perpetuating their proliferation in the environment, thereby creating a potential public health risk. *The E. faecium* CC17 isolated in the hospital wastewater is associated with hospital outbreaks. Nevertheless, information obtained from the study can inform water and public health officials as to the potential health risks and necessity to enhance the removal of ARGs in wastewater

and mitigate their propagation.

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