Prevalence, antibiotic resistance and virulence of *Enterococcus* spp. from wastewater treatment plant effluent and receiving waters in South Africa

L. G. Molale-Tom and C. C. Bezuidenhout IWA

ABSTRACT

Poorly operating wastewater treatment plants (WWTPs) result in faecal pollution of receiving waters, posing a health risk to humans and animals. The aim of this study was to determine the antimicrobial resistance patterns and presence of virulent genes in *Enterococcus* spp. isolated from three WWTPs' final effluent and receiving waters in the North West Province, South Africa. Sixty-three *Enterococcus* spp. were identified and their antimicrobial susceptibility, as well as the presence of five virulence genes, determined. The antibiotic inhibition zone diameter data were subject to cluster analysis. Sixty-eight percent of the screened *Enterococcus* spp. were resistant to three or more antibiotics and harboured plasmids. Five virulence genes were detected and six multi-virulence profiles observed. Cluster analysis indicated groupings of isolates from all three effluent points downstream together, and between plants 1 and 2 together. The findings of this study have demonstrated that *Enterococcus* spp. harbouring virulence factors and plasmids that mediate multiple antibiotic resistance are present in effluent and receiving water systems that support various social needs. This is a cause for concern and it is recommended that *Enterococcus* be used as an additional faecal indicator when microbiological quality of water is assessed.

Key words | *Enterococcus* spp., final effluent, multiple antibiotic resistance, public health, virulence genes

L. G. Molale-Tom (corresponding author)
C. C. Bezuidenhout IWA
Unit for Environmental Sciences and Management,
Microbiology,
North-West University,
Potchefstroom Campus, Private Bag X6001,
Potchefstroom 2520,
South Africa
E-mail: *lesego.molaletom@nwu.ac.za*

HIGHLIGHTS

- Presence of *Enterococcus* species inclusive of *Enterococcus faecium* and *Enterococcus faecalis* in WWTP effluent and downstream.
- Presence of *Enterococcus* species resistant to multiple locally prescribed antibiotics in the clinical setting.
- Presence of *Enterococcus* species harbouring virulence genes not yet reported in South Africa.
- Antibiotic-resistant profiles of *Enterococcus* species before and after curing, illustrating that some of the resistance is plasmid mediated.

INTRODUCTION

In spite of advances and improvements in wastewater treatments, numerous wastewater treatment plants (WWTPs) in South Africa have been reported to be functioning inefficiently (DWA 2012; Iweriebor *et al.* 2015; Hamiwe *et al.* 2019). According to Dąbrowska *et al.* (2017), municipal wastewater effluents contain various toxic substances, such as pharmaceuticals, hormones and pathogens. The standard microbiological indicator parameter for disinfection efficiency in South Africa is *Escherichia coli* (DWA 2013). However, studies have illustrated the presence of

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other microorganisms, in effluent, some of which are potential pathogens (Cai *et al.* 2014; Do *et al.* 2019; Numberger *et al.* 2019). Among these potentially pathogenic microorganisms are the *Enterococcus* group which have been frequently reported in effluents (Iweriebor *et al.* 2015; Taučer-Kapteijn *et al.* 2016; Hamiwe *et al.* 2019; Fouz *et al.* 2020).

Enterococcus are a diverse, complex and important group of bacteria with regard to their interaction with humans. They are commensal organisms in the mammalian gastrointestinal tract but are also found in soil, water and plants (Byappanahalli *et al.* 2012). Despite being harmless *in vivo*, their spread from the intestinal tract to other mucosal and skin surfaces can cause infections (Vu & Carvalho 2011). Enterococci are opportunistic pathogens and target patients with underlying diseases, as well as those treated with a broad spectrum of antibiotics, the elderly and immune-compromised persons (Vu & Carvalho 2011). For *Enterococcus* spp. to cause infection, they must possess virulence factors that allow them to colonize and invade the host's tissue, translocate through epithelial cells and evade the host's immune response (Hammad & Shimamoto 2014). Additionally, the emergence of infections in a susceptible host is determined by the presence of multiple virulence factors, in pathogenic bacteria, functioning collectively or singularly at various stages of infection (Wu *et al.* 2008). Virulence factors are gene products, traits or effector molecules produced by pathogens in order to enhance the ability of a microorganism to cause tissue damage, disease and systematic inflammation (Mundy *et al.* 2000; Vu & Carvalho 2011). For this reason, the study of these organisms' virulence mechanisms has become an important aspect (Semedo *et al.* 2003). Studying these organisms' virulence mechanisms allows for determining their ability to establish infection in a susceptible host (Wu *et al.* 2008).

Additionally, enterococci form part of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) pathogens on the World Health Organisation's priority list (Ramsamy *et al.* 2018). These pathogens have been recognized as common causes of nosocomial infections due to their ability to escape the biocidal action of readily prescribed antibiotics (Hrenović *et al.* 2019). This is because *Enterococcus* spp. have been exposed to multiple antibiotics in the hospital setting, such as (but not limited to) amoxicillin, ampicillin and vancomycin, that have provided them with an evolutionary pressure for selection (SAASP 2014). The resistance of *Enterococcus* spp. to various antibiotics allows them to survive the hospital environment which provides an opportunity for their dissemination (Murray 1990). This dissemination can be via wastewater from domestic, industrial, veterinary and hospital settings; inevitably ending up in municipal WWTPs. Consequently, the WWTPs do not remove pollutants adequately allowing for bacteria, as well as antimicrobial residues, to enter receiving environmental water bodies (Michael *et al.* 2013).

Several studies have reported the presence, emergence, as well as outbreak of antibiotic-resistant clinical *Enterococcus* spp., in South Africa (McCarthy *et al.* 2000; von Gottberg *et al.* 2000), while in recent times, studies have reported the presence of antibiotic-resistant *Enterococcus* pathogens in hospital and domestic wastewater effluents in other parts of the country (Iweriebor *et al.* 2015; Hamiwe *et al.* 2019). However, there is a paucity of data on susceptibility patterns and epidemiology of antibiotic-resistant *Enterococcus* isolated from WWTPs harbouring virulence genes in the North West Province. The presence of *Enterococcus* spp. exhibiting antibiotic resistance and harbouring clinically relevant virulence genes is of particular interest due to the possible link of community-acquired enterococcal infections and recreational activities. The data generated in this study will be relevant and of value to surveillance programmes, such as the WHO Global Antimicrobial Resistance Surveillance System.

The aim of this study was to determine the antimicrobial resistance patterns and presence of virulence genes in *Enterococcus* spp. isolated from three municipal WWTPs' final effluent, as well as receiving environmental water systems in the North West Province, South Africa.

MATERIALS AND METHODS

Sample collection and bacterial isolation

Three WWTPs in the North West Province which receive wastewater from urban households, industries, farms and

hospitals were sampled during May 2015. All samples were collected at the final effluent points downstream and a point between two of the WWTPs. The GPS co-ordinates illustrating the position of the three WWTPs are displayed in Table 1. Three maps indicating the various sampling sites can be found in the Supplementary Appendix. Plant 1 uses a trickling filter system, plants 2 and 3 are activated sludge systems.

The dip sampling technique was employed at each sampling site (US EPA 1994). Briefly, a rope was tied to the neck of the bottle and the bottle was dipped into the surface water system. All water samples were collected aseptically in a sterile glass bottle. Sterilized bottles were labelled before sampling and gloves were worn when handling the bottles. All samples were kept on ice in a sealed cooler box and analysed within 6 h of collection. The membrane filtration technique was employed for *Enterococcus* isolation and enumeration (Greenburg *et al.* 1998). Triplicates of 100 ml water samples were filtered through 0.45 μm (47 mm grid) PALL Corporation sterilized filter membranes [(PALL Life Sciences, Mexico) (cat. no. GN-6 Metricel Membrane 66191)] and placed on KF-*Streptococcus* agar containing 1 ml of 2,3,5-Triphenyltetrazolium chloride per 100 ml (Sigma Aldrich, South Africa). The KF-*Streptococcus* agar plates were incubated at 37 °C for 48 h. Single well-isolated pink colonies were aseptically sub-cultured three times on nutrient agar using the streak plate technique and incubated for 24 h at 37 °C. A total of nine isolates were chosen for each sampling site.

Table 1 | GPS co-ordinates of all sampling sites

		GPS co-ordinates	
WWTP	**Sampling site**	**Longitude (S)**	**Latitude (E)**
Plant 1	P1 final effluent	26°19′14.4″	026°48′13.7″
	P1 point downstream	26° 19′ 24.7″	026°48′10.5″
	Point between plants 1 and 2	26°19′32.7″	026°48′083″
Plant 2	P2 final effluent	26°53′50.0″	026°37′24.3″
	P2 point downstream	26°53′53.5″	026°38′30.4″
Plant 3	P3 plant 3	26°45′05.5″	27°05′40.7″
	P3 point downstream	26°45′48.5″	27°05′25.4″

Enterococcus genomic DNA isolation and identification

For each *Enterococcus* isolate screened, genomic DNA was extracted using a Macherey-Nagel Nucelospin Tissue kit using the manufacturer's instructions (Macherey-Nagel, Germany). The quantity and quality of the isolated total genomic DNA were determined using a NanoDrop™ 1000 Spectrophotometer (Thermo Fischer Scientific, USA) and agarose electrophoresis. The genomic DNA was then amplified using an ICycler thermal cycler (Bio-Rad, UK). A polymerase chain reaction (PCR) reaction mixture with a final volume of 25 μl was used and it contained RNase/DNase free water (Fermentas Life Sciences, USA), 12.5 μl 2× PCR Master Mix (0.05 U/μl Taq DNA Polymerase in reaction buffer, 0.4 mM of each dNTP, 4 mM $MgCl_2$) (Fermentas Life Sciences, USA), 50 μM primer and 1 μl bacterial DNA template (50–100 ng/μl). A portion of 16S small-subunit ribosomal genes positioned 341–907 (V3-V5) was amplified using the primer set described in Muyzer *et al.* (1993). Furthermore, the PCR reaction was set such that initial denaturation occurred at 95 °C for 30 s. Thereafter, the PCR mixture was subjected to 35 cycles of 30 s at 95 °C, 30 s at 52 °C and 60 s at 72 °C, followed by a final extension step of 180 s at 72 °C. All PCR amplifications were confirmed using electrophoresis.

16S rRNA sequencing

PCR products were purified using ZR DNA Sequencing Clean-up Kit (Zymo Research, USA) and as described by Li *et al.* (2010). The partial 16S rRNA gene was sequenced and analysed as described in Jordaan & Bezuidenhout (2013). A total of 63 *Enterococcus* spp. nucleotide sequences were submitted to the GenBank database under accession numbers KT598397-KT598459.

Detection of virulence genes using PCR

Five oligonucleotide primer pairs (Applied Biosystems, UK) used for the detection of virulence genes were obtained from Vankerckhoven *et al.* (2004). Identification of virulence genes for each isolate was performed by separate PCR for the virulence genes *asa1*, *hyl* and *gelE* while a duplex reaction was used for *cylA* and *esp*. PCR amplifications were

performed in a Techne Prime Elite thermocycler (Cambridge, UK), in 0.2 ml reaction tubes. PCR assays were performed in a total volume of 25 µl containing 1 µl bacterial DNA template (50–100 ng/µl) for the single assays and 2 µl bacterial DNA template (100–150 ng/µl) for the duplex reactions. Additionally, the reaction contained RNase/DNase free water (Fermentas Life Sciences, USA), 2× DreamTaq PCR Master Mix (0.05 U/µL Taq DNA polymerase in reaction buffer, 0.4 mM of each dNTP and 4 mM $MgCl_2$), 0.2 µM of primers *asa1* and *gelE* and 0.4 µM of primers *cylA*, *esp* and *hyl*. The PCR cycling conditions consisted of denaturing at 95 °C for 180 s, followed by 30 cycles of 95 °C for 30 s, annealing at 56 °C for 30 s and 72 °C for 60 s. This was followed by a final step of 72 °C for 600 s. PCR products were confirmed by gel electrophoresis.

Antimicrobial susceptibility testing

Antimicrobial susceptibility patterns of *Enterococcus* isolates were determined using the disk diffusion method (Bauer *et al.* 1966). Assays were performed on Mueller Hinton agar (Merck, Germany) using ampicillin (10 µg), amoxicillin (10 µg), penicillin G (10 µg), neomycin (30 µg), streptomycin (300 µg), vancomycin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), tetracycline (30 µg), trimethoprim (2.5 µg) and erythromycin (15 µg). All antibiotics were obtained from Mast Diagnostics (UK). *Enterococcus* isolates were classified resistant, susceptible or intermediate according to the criteria from the Clinical and Laboratory Standards Institute (CLSI 2017).

Plasmid isolation and curing

Pure *Enterococcus* isolates showing resistance to two or more antibiotics were grown on nutrient agar, cultured overnight at 37°C in 20 ml Brain Heart Infusion broth (BHI, Merck, Germany) and harvested by centrifugation. A plasmid isolation kit was used to extract total plasmids according to the manufacturer's instructions (Macherey-Nagel, Germany). All plasmid products were electrophoresed for 1 h at 60 V and agarose gel images were captured under a UV light using a Bio-Rad ChemiDoc imaging system (Hercules, CA). *Enterococcus* isolates resistant to two or more antibiotics were subjected to plasmid curing according to Molina-Aja *et al.* (2002) and Carvalho *et al.* (2014). The isolates were once again subjected to antibiotic susceptibility testing and resistance classified as plasmid dependent when affected by curing.

Statistical analysis

The inhibition zone diameter data of *Enterococcus* spp. were subject to cluster analysis using Ward's method and Euclidean distances in Statistica 12.0 (StatSoft, USA).

RESULTS

Enterococcus levels

In Table 2, the triplicate average values of presumptive *Enterococcus* spp. And ±standard deviation values are presented. The average presumptive *Enterococcus* levels ranged from 52.00 to >315.00 cfu/100 ml. The highest levels were observed at plant 2 final effluent (>315.00 ± 0.00 cfu/100 ml), whereas the lowest values were observed at plant 3 point downstream (52.00 ± 1.73 cfu/100 ml).

Identification of *Enterococcus* spp.

The most frequently detected *Enterococcus* spp. were *E. hirae* (21%), *E. faecalis* (21%) and *E. faecium* (19%). The remaining detected species were *E. gallinarum* (13%), *E. casseliflavus* (16%), *E. mundtii* (8%) and *E. durans*

Table 2 | Presumptive *Enterococcus* levels per sampling site

WWTP	Sampling site	*Enterococcus* spp. cfu/100 ml
Plant 1	P1 final effluent	113.33 ± 2.88
	P1 point downstream	116.00 ± 0.82
	Point between plants 1 and 2	110.66 ± 2.08
Plant 2	P2 final effluent	>315.00 ± 0.00
	P2 point downstream	198.66 ± 0.58
Plant 3	P3 plant 3	82.33 ± 2.52
	P3 point downstream	52.00 ± 1.73

Triplicate average values of presumptive *Enterococcus* spp. and ±standard deviation values are presented.

(3%). All *Enterococcus* species were identified to a sequence similarity threshold of >97%.

The antibiotic inhibition zone diameter data from the antimicrobial susceptibility test performed on all 63 *Enterococcus* spp. were subjected to cluster analysis which is presented in Figure 1. Two main clusters, A and B, were generated and cluster A sub-divided into minor clusters A1 and A2. Furthermore, the species composition of both clusters was determined and is presented in Table 3. Differences in species diversity and prevalence levels at the various sites were observed. Furthermore, as depicted in Figure 1, the cluster analysis indicated grouping of the antibiotic inhibition zone results of *Enterococcus* spp. isolated at the final effluent and points downstream, respectively. Cluster A1 comprised *Enterococcus* spp. from all sampled effluent. This cluster was predominated by *E. faecalis* isolates followed by *E. faecium* and *E. hirae* isolates (Table 3). Furthermore, as depicted in Figure 1, cluster A2 comprised *Enterococcus* spp. isolated from the points downstream the effluents.

The predominant species in this cluster (A2) were *Enterococcus gallinarum, E. casseliflavus* and *E. mundtii* (Table 3). Lastly, cluster B represented *Enterococcus* isolates from the point located between plants 1 and 2. In this cluster, the predominant species, as presented in Table 3, were *E. faecium* and *E. hirae*.

Identification of virulence genes and report of multiple antibiotic resistance phenotypes prior to and post-plasmid curing

Presented in Table 4 are the characteristics of *Enterococcus* spp. isolated from three WWTPs' final effluent and points downstream with reference to the presence of virulence genes, as well as the MAR phenotypes prior to and post-plasmid curing.

Virulence

Figure 2 is an agarose gel with representative isolates confirming the presence of virulence genes in some *Enterococcus* spp. screened in this study. All five virulence genes screened were detected and *Enterococcus* isolates harbouring virulence genes are presented in Table 4. As presented in Table 4, the most frequently detected virulence determinants were *esp* (25%, 10/39), *hyl* (25%, 10/39), *gelE* (23%, 9/39) and *cylA* (17%, 7/39). Nonetheless, *asa1* (7%, 3/39) was also detected though at lower prevalence levels. *Enterococcus faecium* spp. predominantly harboured the *gelE* (55%, 5/9) and *hyl* (3%, 3/10) genes (Table 4). The *esp* gene was predominantly harboured by *E. faecalis*, *E. casseliflavus* and *E. hirae* spp. at prevalence levels of 3% (3/10) each. Furthermore, virulence gene *cylA* was predominantly detected in *E. faecalis* and *E. gallinarum* spp. at prevalence levels of 29% (2/7) each (Table 4). In addition, six multi-virulence profiles were observed with *cylA–esp* being the most prominent.

Antibiotic resistance

Analysis of the antimicrobial susceptibility of the 63 *Enterococcus* isolates revealed that resistance to ampicillin (67%), vancomycin (62%), tetracycline (58%), penicillin (52%) and erythromycin (51%) was most frequent. Furthermore, resistance to chloramphenicol (22%) and streptomycin (17%) was also observed, although at slightly lower levels. Additionally, low frequencies of resistance to ciprofloxacin (8%) and gentamycin (6%) were detected.

Sixty-eight percent of the screened *Enterococcus* spp. were resistant to three or more antibiotics (Table 4), while resistance to multiple antibiotics was more common among the *E. faecium* isolates followed by *E. faecalis* and *E. casseliflavus* spp. As depicted in Table 4, a total of 19 multiple antibiotic resistance (MAR) phenotypes were identified with the most frequently identified MAR phenotype being ERY–STREP–PEN–TET. The latter MAR phenotype was observed in nine *Enterococcus* isolates. In addition, AMP–PEN–TET–VA was the second-most prevalent MAR phenotype and was identified in six *Enterococcus* isolates screened. Similarities in species composition of these MAR phenotypes were observed as they were composed of *E. faecalis, E. faecium, E. casseliflavus* and *E. gallinarum* spp. (Table 4).

Seventy-six percent (35/46) of the *Enterococcus* isolates resistant to two or more antibiotics had plasmids. The plasmids were of high-molecular weight. Furthermore, in Table 4, MAR phenotypes of the screened *Enterococcus* spp. pre- and post-plasmid curing are presented. Differences

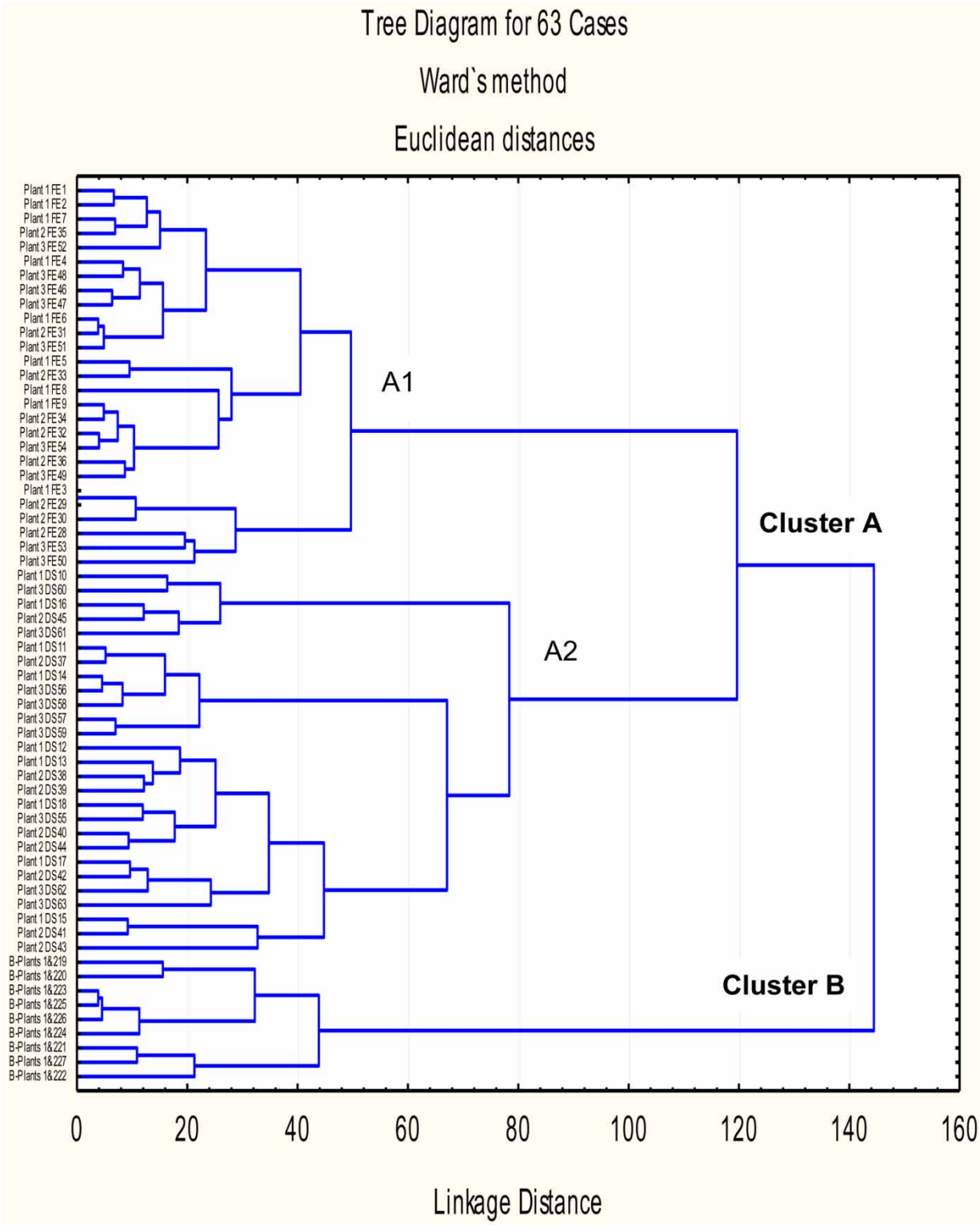

Figure 1 | Dendrogram showing the relationship of 63 *Enterococcus* spp. obtained from three WWTPs' final effluent and points downstream, based on inhibition zone diameter data (FE: final effluent, DS: downstream the final effluent, B-plants 1 & 2: between plants 1 and 2).

Table 3 | *Enterococcus* species composition of clusters A and B

	Cluster A		
Species	**Minor cluster A1**	**Minor cluster A2**	**Cluster B**
	$N = 27$	$N = 27$	$N = 9$
E. faecalis	9 (33%)	4 (15%)	0
E. faecium	6 (22%)	2 (7%)	4 (44%)
E. hirae	6 (22%)	4 (15%)	3 (33%)
E. gallinarum	1 (4%)	7 (26%)	0
E. casseliflavus	4 (14%)	5 (18.5%)	1 (11%)
E. mundtii	0	5 (18.5%)	0
E. durans	1 (4%)	0	1 (11%)

in MAR phenotypes were observed for all profiles pre- and post-plasmid curing. Thirty-three percent (10/30) of the *Enterococcus* spp. resistant to vancomycin were still resistant to this antibiotic after plasmid curing, while resistance to penicillin, ampicillin, erythromycin and chloramphenicol was observed in few isolates post-plasmid curing (Table 4).

DISCUSSION

Although antibiotic resistance profiles have often been detected in recreational and wastewaters (Soge *et al.* 2009; Araújo *et al.* 2010), the current knowledge on the prevalence of antibiotic-resistant bacteria and types of antibiotic resistance in the environment is insufficient (Rizzo *et al.* 2013). Urban WWTPs provide environmental waters, used for various agricultural, religious, cultural and recreational activities and effluent for reuse. However, ineffectively functioning plants are among the main sources of antibiotics released into the environment. This contamination of environmental surface water systems with antibiotics consequently facilitates the selection of antibiotic-resistant genes and antibiotic-resistant bacteria (Martinez 2009) which is a concern when the health and well-being of both humans and animals are considered.

In this study, 63 *Enterococcus* spp. isolated from the final effluent of three municipal WWTPs and receiving environmental water systems were screened for antimicrobial resistance and clinically relevant virulence genes. Differences in species composition and diversity at varying prevalence levels were observed for the various sites. Graves & Weaver (2010) explain that the observed differences are a result of influences exerted by environmental factors, as well as the sources of a sewer system. Carvalho *et al.* (2014) reported higher species diversity in raw sewage compared to sites more distant from the raw sewage. The latter findings support the findings of this study where species diversity was higher at the final effluent sites as compared to the site between plants 1 and 2. Furthermore, similar to the findings of other studies, *E. hirae*, *E. faecalis* and *E. faecium* were the most frequently identified *Enterococcus* spp. (da Silva *et al.* 2006; Łuczkiewicz *et al.* 2010).

The high prevalence of *E. hirae* isolates identified in this study correlates with the findings of Bonilla *et al.* (2006) and da Silva *et al.* (2006), who reported high *E. hirae* levels in recreational and urban wastewater. In addition, studies have also reported the dominance of *E. hirae* species in animal faeces (Fisher & Phillips 2009; Peeters *et al.* 2013). Thus, the high levels of *E. hirae* spp. identified in this study suggest faecal contamination. The presence of *E. hirae* spp. in the WWTPs' final effluent and points downstream used for recreational, agricultural and cultural activities is a cause for concern since several studies have reported the ability of *E. hirae* to cause wound infections, gastritis and bacteraemia (Talarmin *et al.* 2011; Chan *et al.* 2012).

Enterococcus faecalis and *E. faecium* were detected in the WWTPs' final effluent. This is in accordance with the findings of several other studies (Cheng *et al.* 2012; Varela *et al.* 2013; Iweriebor *et al.* 2015). Finding *E. faecalis* and *E. faecium* at high levels in all three sampled effluents suggests poorly disinfected sewage effluent as these two species are the most commonly found *Enterococcus* spp. in the human gastrointestinal tract (Manero *et al.* 2002; Klein 2003). Cheng *et al.* (2012) suggested that frequently detecting *E. faecalis* and *E. faecium* in treated effluent may imply that these species are naturally present at higher concentrations in the influent or may be more resistant to treatment processes, particularly disinfection. Furthermore, their presence in effluent and points downstream may suggest the dissemination of these species from the WWTP into the environment. Their presence in

Table 4 | Virulence and antimicrobial-resistant characteristics of *Enterococcus* spp. isolated from three WWTP effluents and points downstream

			Antimicrobial resistance profiles	
Isolate	**Species**	**Virulence gene**	**Before plasmid curing**	**After plasmid curing**
VE1	*E. casseliflavus*	*Esp*	ERY–VA	–
VE5	*E. faecium*	*gelE*		VA
VE2	*E. casseliflavus*	–	CHLOR–PEN–VA	–
VE4	*E. faecium*	*cylA, esp, gelE*	AMP–ERY–PEN–TET–VAN	VA
KE31	*E. faecalis*	*cylA, esp*		–
VE7 H	*E. hirae*	–	AMP–ERY–PEN–CIP–VA	AMP–PEN
VE8 F	*E. faecalis*	*cylA, esp*		–
VDS10	*E. casseliflavus*	–	AMP–ERY–PEN–GEN–TET–VA	AMP–PEN
VDS11	*E. mundtii*	*asa1*	ERY–STREP–PEN–TET	–
VDS14	*E. faecalis*	*gelE*		–
OV19	*E. faecium*	–		–
OV20	*E. faecium*	–		–
KDS37	*E. casseliflavus*	*esp*		–
PDS56	*E. gallinarum*	*hyl*		–
PDS57	*E. gallinarum*	*cylA, esp*		–
PDS58	*E. gallinarum*	–		–
PDS59	*E. gallinarum*	–		–
VDS12	*E. hirae*	–	AMP–PEN–TET	–
OV21	*E. casseliflavus*	*hyl*		–
OV24	*E. gallinarum*	*cylA*		–
VDS13	*E. faecalis*	–	AMP–TET–VA	–
VDS15	*E. faecium*	*Hyl*	AMP–STREP–TET–VA	–
PE46	*E. faecium*	–		–
VDS16	*E. faecium*	*gelE*	AMP–CHLOR–ERY–PEN–GEN–TET–VA	PEN
OV22	*E. hirae*	*esp, hyl*	AMP–PEN–TET–VA	–
OV23	*E. hirae*	*asa1, gelE*		–
OV25	*E. faecium*	–		–
OV26	*E. faecium*	*hyl, gelE*		–
KE33	*E. casseliflavus*	*Hyl*	AMP–ERY–TET–VA	–
KE36	*E. faecalis*	–		–
KDS41	*E. gallinarum*	–		–
KDS45	*E. gallinarum*	–		VA
PE46	*E. faecium*	–		–
PE47	*E. faecalis*	*Hyl*		ERY–TET
KE32	*E. hirae*	*Hyl*	AMP–ERY–VAN	VA
KE34	*E. faecalis*	*cylA, esp*		–
PE51	*E. faecium*	*asa1*		VA
KE35	*E. faecium*	*gelE*	AMP–PEN–CIP–VA	VA

(*continued*)

Table 4 | continued

			Antimicrobial resistance profiles	
Isolate	**Species**	**Virulence gene**	**Before plasmid curing**	**After plasmid curing**
KDS38	*E. casseliflavus*	*cylA, esp, gelE*	AMP–VA	VA
KDS40	*E. casseliflavus*	–	AMP–ERY–TET	VA
PE48	*E. hirae*	*esp, gelE*	ERY–PEN–TET–VA	TET-VA
PE52	*E. faecalis*	*Hyl*	AMP–CHLOR–CIP–TET–VA	–
PE53	*E. faecalis*	*Hyl*	CHLOR–TET–VA	–
PDS60	*E. mundtii*	–	AMP–CHLOR–ERY–GEN–TET	–
PDS61	*E. mundtii*	–		VA
PDS62	*E. mundtii*	–	AMP–CHLOR–PEN	CHLOR

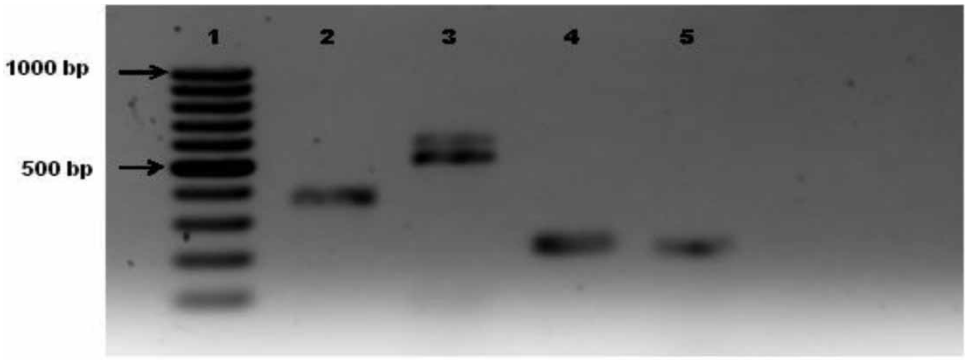

Figure 2 | A 1% (w/v) agarose gel image of four representative *Enterococcus* isolates harbouring the five virulence genes of interest [Lane 1: 100 bp molecular weight marker, 2: *asa1* (375 bp), 3: *esp* (510 bp) & *cyl*A (688 bp), 4: *hyl* (276 bp), 5: *gelE* (213 bp)].

the effluent is also a cause for concern seeing that they account for a majority of infections caused by *Enterococcus* spp. (Vu & Carvalho 2011).

Enterococcus gallinarum, *E. casseliflavus* and *E. mundtii* were also isolated in this study and were the most prevalent isolates in surface water bodies receiving the final effluent from the WWTPs. The presence of these species in the downstream sites is not unexpected due to the well-documented presence of these *Enterococcus* species in plants, soil and water non-human animal hosts (Byappanahalli *et al.* 2012; Ran *et al.* 2013). However, their presence in downstream sites of receiving water bodies that are used for various social activities is a cause for concern as *E. gallinarum*, *E. casseliflavus* and *E. mundtii* have been listed among *Enterococcus* spp. known to occasionally cause human infection (Perio *et al.* 2006). Thus, implications for the immuno-compromised could have far-reaching effects.

Enterococcus isolates screened in this study displayed the presence of multi-virulence profiles. This is in accordance with the findings of Poeta *et al.* (2005) and Lata *et al.* (2009). Furthermore, the findings of Lata *et al.* (2009) showed that *Enterococcus* isolates exhibiting two to three virulence-markers per isolate were mostly associated with polluted sites.

The *esp* gene was the predominant virulence gene identified and was harboured by *E. faecalis*, *E. casseliflavus*, *E. hirae* and *E. faecium* isolates. Enterococcal surface protein (*esp*) is considered a pathogenicity marker in *E. faecium* and *E. faecalis* clinical isolates (Said *et al.* 2015). Therefore, the predominance of *esp* in *Enterococcus* isolates

obtained from the final effluent and receiving water bodies is not strange as the WWTPs receive wastewater from the hospitals. Furthermore, the presence of *esp* in *Enterococcus* spp. other than *E. faecalis* and *E. faecium* has not been extensively reported. However, it should be noted that its presence in other *Enterococcus* spp. may possibly be a result of genetic exchange by conjugation (Oancea *et al.* 2004).

The *cylA*, *gelE* and *hyl* virulence genes were also detected in the various *Enterococcus* spp. This observation is supported by various studies, conducted in most parts of the world, that reported the presence of these virulence genes in various *Enterococcus* spp. isolated from different sources (Semedo *et al.* 2003; Gonçalves *et al.* 2011; Sidhu *et al.* 2014). However, a recent study in South Africa reported the absence of virulence genes *cylA*, *esp* and *hyl* in *Enterococcus* spp. isolated from WWTP in the Eastern Cape (Iweriebor *et al.* 2015). However, a similar study reported on the presence of *asa* and *esp* in *Enterococcus* spp. isolated from WWTP in Gauteng. Thus, in the present study, we report on virulence patterns previously observed. However, here we also report on some virulence patterns not previously reported, particularly for South African *Enterococcus* spp.

High levels of multiple antibiotic-resistant *Enterococcus* spp. were isolated in this study. This is in accordance with the findings of several other studies that also isolated large numbers of *Enterococcus* from water sources that were resistant to multiple antibiotics (Da Costa *et al.* 2006; Carvalho *et al.* 2014). Furthermore, the observed resistance to vancomycin, tetracycline, penicillin and erythromycin is in agreement with the findings of other studies (Moore *et al.* 2010; Carvalho *et al.* 2014; Iweriebor *et al.* 2015). On the other hand, resistance to ampicillin, chloramphenicol and streptomycin was also observed in the present study. Resistance to the latter antibiotics may not be surprising as these antibiotics are frequently prescribed at South African Hospitals (NDoH 2012).

Similar to the findings of Xu *et al.* (2007) and Carvalho *et al.* (2014), 21 distinct MAR profiles were observed. The presence of *Enterococcus* spp. in wastewater and receiving water systems that are resistant to multiple antibiotics is a cause for concern as these species are associated with both community and hospital-acquired infections. If these antibiotics are commonly used for the treatment of infections (Xu *et al.* 2007), then it could be a further health burden as treatment failure could arise.

However, 67% of the *Enterococcus* spp. with MAR was susceptible to the same antibiotics after plasmid curing, implying that the resistance is plasmid mediated. Similar results were observed by Amaechi (2015) and Carvalho *et al.* (2014). According to McBride *et al.* (2007), the presence of plasmids is common in the *Enterococcus* genus, as these mobile genetic elements constitute a substantial fraction of their genome and are responsible for horizontal gene transfer. Therefore, *Enterococcus* spp. may acquire antimicrobial-resistant genes through mutation or horizontal transfer of mobile genetic elements, such as plasmids and transposons (Hasmann *et al.* 2005). Furthermore, environmental water systems polluted by sewage may act as vehicles of bacteria carrying and disseminating antibiotic-resistant plasmids (Carvalho *et al.* 2014).

Although only one plasmid with a high-molecular weight was observed in all *Enterococcus* spp. screened, studies by Szczepanowski *et al.* (2004) and Schlüter *et al.* (2003) demonstrated that antibiotic resistance genes were commonly contained in high-molecular weight plasmids. Therefore, the presence of *Enterococcus* spp. harbouring plasmids in final effluent flowing into receiving water systems implies that these aquatic systems could potentially be acting as reservoirs of antibiotic-resistant *Enterococcus* harbouring high-molecular weight plasmids.

CONCLUSIONS

In this study, the antimicrobial resistance patterns and presence of virulence genes in *Enterococcus* spp. isolated from three municipal WWTPs' final effluent, as well as receiving environmental water systems in the North West Province, South Africa, were determined. Plant 2 had the highest levels of *Enterococcus* spp. while the point downstream Plant 3 had the lowest levels. Cluster analysis grouped *Enterococcus* spp. according to isolates detected in all three plants' effluent, the point between plants 1 and 2 and the points downstream, respectively. The predominant species identified in the sampled effluent sites were *Enterococcus faecalis*, *E. faecium* and *E. hirae*. The point between plants 1 and 2 was dominated by *Enterococcus gallinarum*, *E. casseliflavus* and *E. mundtii*, while the predominant species in the

downstream sites were *Enterococcus faecium* and *E. hirae*. All five virulence factors screened were identified in this study. Over 60% of the screened *Enterococcus* spp. was resistant to three or more antibiotics with the most prevalent MAR phenotype being ERY–STREP–PEN–TET. Additionally, differences were observed in MAR phenotypes pre- and post-plasmid curing. The findings of this study have demonstrated that *Enterococcus* spp. harbouring plasmids that mediate MAR are not effectively removed during treatment consequently entering environmental water systems that support a variety of social needs. Furthermore, these *Enterococcus* spp. harbour virulence factors, which play a significant role in the rupture of a variety of target membranes, colonization and biofilm production, as well as the catabolism of various target molecules, such as gelatine, collagen, fibrinogen, casein and insulin. Therefore, the data presented here serve as an alert for public health authorities. It is recommended that interventions, such as advanced treatment technologies and disinfection processes, be implemented in WWTPs while environmental water systems used by humans and animals be tested regularly for *Enterococcus* levels.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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