Environ Sci Pollut Res (2016) 23:21501–21510
DOI 10.1007/s11356-016-7369-7

RESEARCH ARTICLE

Antibiotic resistance, efflux pump genes and virulence determinants in *Enterococcus* spp. from surface water systems

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Received: 14 April 2016 / Accepted: 1 August 2016 / Published online: 11 August 2016

Abstract The aim of this study was to report on antibiotic susceptibility patterns as well as highlight the presence of efflux pump genes and virulence genetic determinants in *Enterococcus* spp. isolated from South African surface water systems. One hundred and twenty-four *Enterococcus* isolates consisting of seven species were identified. Antimicrobial susceptibility testing revealed a high percentage of isolates was resistant to β-lactams and vancomycin. Many were also resistant to other antibiotic groups. These isolates were screened by PCR, for the presence of four efflux pump genes (*mefA*, *tetK*, *tetL* and *msrC*). Efflux genes *mefA* and *tetK* were not detected in any of the *Enterococcus* spp. However, *tetL* and *msrC* were detected in 17 % of the *Enterococcus* spp. The presence of virulence factors in the *Enterococcus* spp. harbouring efflux pump genes was determined. Virulence determinants were detected in 86 % of the *Enterococcus* spp. harbouring efflux pump genes. Four (*asa1*, *cylA*, *gel* and *hyl*) of the five virulence factors were detected. The findings of this study have demonstrated that *Enterococcus* from South African surface water systems are resistant to multiple antibiotics, some of which are frequently used for therapy. Furthermore, these isolates harbour efflux pump genes coding for resistance to antibiotics and virulence factors which enhance their pathogenic potential.

Responsible editor: Robert Duran

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Keywords Surface water systems · *Enterococcus* spp. · Multiple antibiotic resistance · Efflux pump genes · Virulence genes

Introduction

There are concerns about the occurrence of bacteria harbouring antibiotic resistance and virulence genes in recreational waters as well as the risk that these may pose to users (Santiago-Rodriguez et al. 2013). Among the environmental bacteria are the enterococci, a bacterial group ubiquitously found in the gastrointestinal tract of humans and animals as well as soil, water and plants (Teixeira and Merquior 2013). Some strains in this bacterial group have been identified as opportunistic pathogens and important etiological agents of nosocomial infections (Top et al. 2008). Consequently, the presence of enterococci in environmental water sources is of particular interest due to the possible link of community acquired infections and recreational activities (APHA-AWWA-WEF 1998).

Enterococci are known to have intrinsic resistance traits while also possessing specific acquired mechanisms of resistance to different antibiotics (Aslangul et al. 2006). These acquired mechanisms of resistance include spontaneous mutation as well as genetic exchange with other bacteria in the environment via horizontal gene transfer. Additionally, coupled to their intrinsic resistance to a variety of antibiotics, enterococci are inherently more resistant to various antimicrobials in comparison to most gram-positive bacteria (Li and Nikaido, 2009). Therefore, this allows for the reasonable assumption that the presence of genes encoding multidrug resistance efflux pumps could be contributing to antimicrobial resistance (Jonas et al. 2001).

Efflux pumps are transporter proteins harboured in both gram-positive and gram-negative bacteria (Bambeke et al.

2000). These transporter proteins extrude various toxic substances including antibiotics from within a cell to its external environment (Webber and Piddock 2003). The mechanism of efflux has previously been studied in enterococci particularly for the efflux of fluoroquinolones as well as chloramphenicol (Lynch et al. 1997). Furthermore, 34 efflux pump genes have been identified in the genome of *Enterococcus faecalis* spp. (Davis et al. 2001; Jonas et al. 2001).

According to Teixeria and Merquior (2013), *Enterococcus* spp. are among the leading therapeutic challenges with regard to life-threatening infections and are becoming significant pathogens worldwide. Infections caused by *Enterococcus* spp. are frequently treated with tetracyclines (Santiago-Rodriguez et al. 2013). However, erythromycin and other macrolides are also generally used for enterococcal infections, particularly where allergy to β-lactams is suspected (Arvanitidou et al. 2001; Duarte et al. 2005).

Nevertheless, antibiotic resistance alone cannot explain the pathogenicity of this bacterial group. *Enterococcus* spp. may possess genes coding for virulence factors and their protein products which contribute to the species infection potential (Hill 2012). Various virulence factors such as aggregation substance, cytolysin, enterococcal surface protein, gelatinase and hyaluronidase have been considered and could play a role in rendering *Enterococcus* spp. pathogenic (Lata et al. 2009). Moreover, previous studies have demonstrated that *Enterococcus* spp. carrying antibiotic resistance genes can harbour genes encoding virulence factors (Shankar et al. 2001).

Recent studies have contributed to the understanding of the prevalence of antibiotic resistance and virulence genes in *Enterococcus* spp. isolated from human and animal sources (Sidhu et al. 2014). Several studies have reported the presence, emergence as well as outbreak of antibiotic resistant enterococci in South Africa (Budavari et al. 1997; Struwig et al. 1998; McCarthy et al. 2000; von Gottberg et al. 2000). However, similar studies on enterococci isolated from surface water and other environmental sources are limited.

The goal of the present study was thus to determine the prevalence of *Enterococcus* spp. in South African surface water systems, their antibiotic susceptibility patterns as well as the presence of efflux pump genes coding resistance to antibiotics. Furthermore, virulence factors of isolates harbouring antibiotic resistant genes are reported. In this way, the importance of enterococci in water quality assessments was demonstrated.

Materials and methods

Study area and sample collection

The study area, depicted in Fig. 1, comprised a total of four Rivers (Vaal, Harts, Schoonspruit and Mooi) and an inland lake (Barberspan) flowing in and through the North West Province (NWP) of South Africa. These surface water resources are important to the province as they largely support the gold and platinum mining, manufacturing industries, agricultural sector as well as urban populations. Surface water samples were collected between March 2010 and August 2011. The direct and dip sampling techniques were employed depending on the physical setting of each sampling site (US EPA 1994).

Enumeration and isolation of *Enterococcus*

Membrane filtration was used for *Enterococcus* isolation and enumeration (US EPA, 2000). Triplicates of 100 ml water samples were filtered through 0.45 μm (47 mm grid) PALL Corporation sterilized filter membranes (PALL Life Sciences, Mexico) and placed on KF-Streptococcus agar containing 1 ml of 2,3,5-Triphenyltetrazolium chloride (TTC) 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. Streaking was done three times in order to ensure that pure cultures were obtained.

Genomic *Enterococcus* DNA isolation and identification

Pure *Enterococcus* isolates 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 commercial genomic DNA isolation kit, Genomic DNA from tissue (Macherey-Nagel, Germany), was used to isolate total DNA, using the instructions of the manufacturer. The quantity and quality of the isolated total genomic DNA were determined using a NanoDrop TM 1000 Spectrophotometer (Thermo Fischer Scientific, US) and agarose electrophoresis. The 16S rDNA (Table 1) was then amplified using an ICycler thermal cycler (Bio-Rad, UK). Reagents and procedures for the PCR and evaluation of amplification success are described by Jordaan and Bezuidenhout (2013). The annealing temperature in this case was 52 °C. Amplicons were sequenced by Inqaba Biotech (South Africa, Pretoria). Raw sequence data was transferred to Geospiza Finch TV (version 1.4) software which was used to view all chromatograms. All amplified DNA sequences were identified using BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST).

Antimicrobial susceptibility testing

Antimicrobial susceptibility patterns of *Enterococcus* isolates were determined using the disc diffusion method (Bauer et al. 1966; CLSI 2012). Assays were performed on Mueller Hinton agar (Merck, Germany) using ampicillin (10 μg), amoxicillin

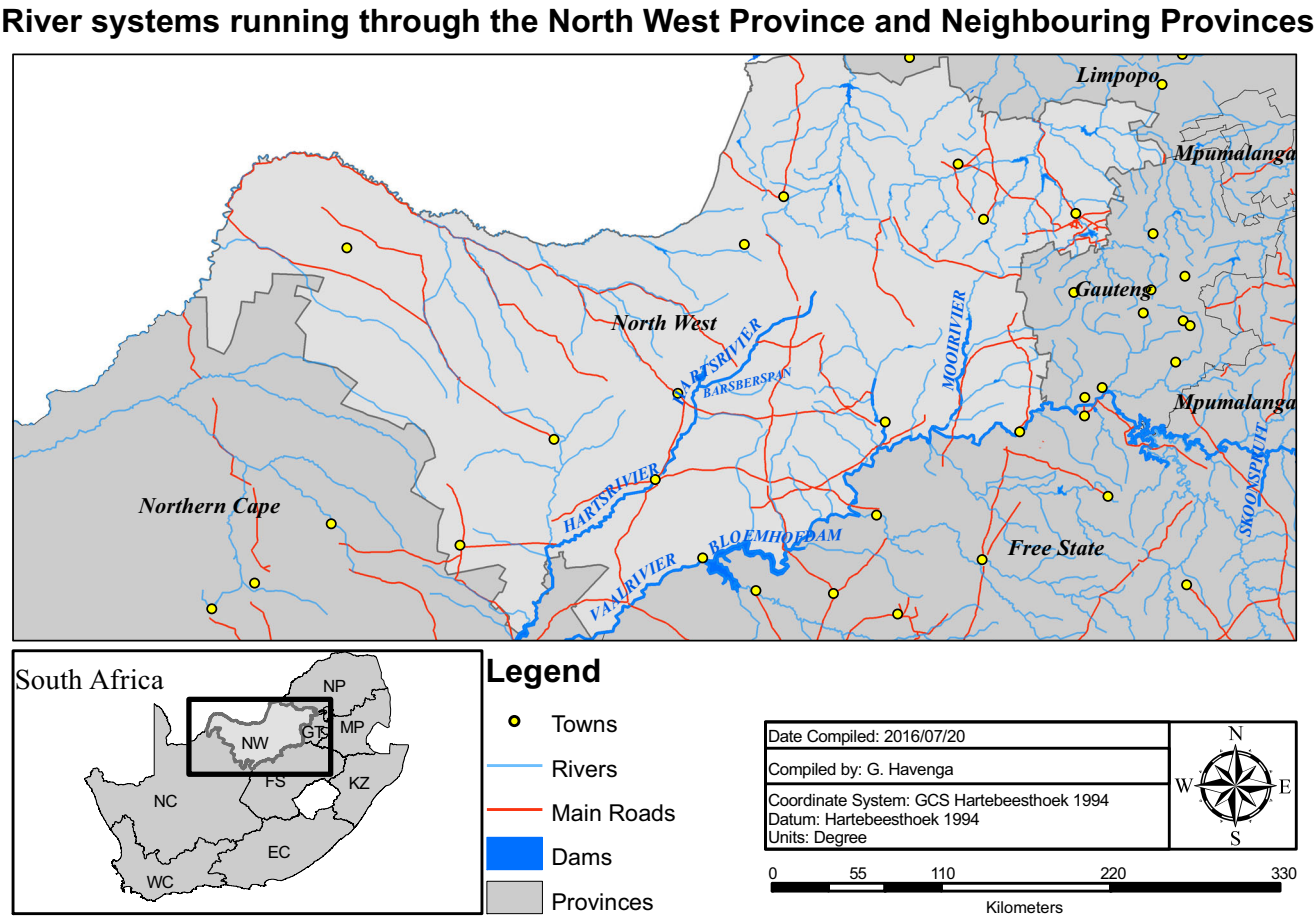

Fig. 1 Map illustrating the spatial distribution and geographical location of the various surface water systems of interest

(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 Clinical and Laboratory Standards Institute (CLSI 2012).

PCR detection of efflux pump genes

Four efflux pump genes were targeted (Table 1) using the primers (Alied Biosystems, UK) in Table 1. The DNA extracted for genomic identification was used to determine the presence of efflux pump genes. The final reaction volumes were 20 μl (*msrA/B*, *tetK* and *tetL*) and 25 μl (*mefA*). The PCR reaction for *msrA/B* contained 1 μl DNA template (30–50 ng/μl), 6 μl RNase/DNase free water (Fermentas Life Sciences, US), 12.5 μl 2× DreamTaq PCR Master Mix (0.05 U/μL *Taq* DNA polymerase in reaction buffer, 0.4 mM of each dNTP and 4 mM $MgCl_2$), 0.5 μl (0.25 μM) of each primer.

For detection of *tetK* and *tetL*, 1 μl bacterial DNA template (50–100 ng/μl), 6.1 μl RNase/DNase free water (Fermentas Life Sciences, USA), 12.5 μl 2× DreamTaq PCR Master Mix (0.05 U/μL Taq DNA polymerase in reaction buffer, 0.4 mM of each dNTP and 4 mM $MgCl_2$), 0.4 μl (0.2 μM) of each primer was used. Reaction mixtures for the detection of the *mefA* gene, contained 1 μl DNA template (30–50 ng/μl), 7.1 μl RNase/DNase free water (Fermentas Life Sciences, US), 3.2 μl $MgCl_2$ (4 mM), of each 2.5 μl deoxynucleotide triphosphate (0.2 mM), 0.2 μl *Taq* DNA polymerase (0.5 U/μl), 4 μl Tris-HCL (200 mM), 1 μl KCL (500 mM) and 1 μl of each primer (0.5 μM). The PCR cycling conditions of all primers are described in Table 1.

PCR detection of virulence genes

Five oligonucleotide primer pairs (Applied Biosystems, UK) were used for the detection of virulence genes and were obtained from Vankerckhoven et al. (2004) (Table 1). DNA extracted for genomic identification was used to detect the presence of virulence genes. Polymerase chain reaction (PCR) was used for the identification of all virulence genes. The reaction mixtures, in final volumes of 25 μl, for detection of the various virulence genes contained 50–100 ng/μl bacterial DNA and reaction mix was the same as for

Table 1 Oligonucleotide primers used in this study

	Target/ Primer	Sequence (5′-3′)	Size (bp)	PCR conditions	Reference
16S rRNA gene	*16S RNA*-F *16S RNA*-R	CCTACGGGAGGCAGCAG CCGTCAATTCCTTTGAGTTT	550	Denaturation 95 °C 300 s, 35 cycles at 95 °C 30 s, 52 °C 30 s,72 °C 60 s, 72 °C 180 s	Muyzer et al. (1993)
Efflux pump genes	*mefA-F* *mefA-R*	AGTATCATTAATCACTAGTGC TTCTTCTGGTACTAAAAGTGG	348	Denaturation 94 °C 180 s, 35 cycles at 93 °C for 60 s, 52 °C 30 s, 72 °C 60 s, 72 °C 300 s	Duarte et al. (2005)
	tetK-F *tetK-R*	TATTTTGGCTTTGTATTCTTTCAT GCTATACCTGTTCCCTCTGATAA	1159	Denaturation 95 °C 60s, 35 cycles at 50 °C 60 s, 72 °C 30 s, 72 °C at 300 s	
	tetL-F *tetL-R*	ATAAATTGTTTCGGGTCGGTAT AACCAGCCAACTAATGACAATGAT	1,1077	Denaturation 95 °C 60 s, 35 cycles at 50 °C 60 s, 72 °C 30 s, 72 °C at 300 s	
	msrA/B-F *msrA/B-R*	GCAAATGGTGTAGGTAAGACAACT ATCATGTGATGTAAACAAAAT	400	Denaturation 95 °C 180 s, 35 cycles at 93 °C 30 s, 55 °C 120 s, 72 °C 90 s	Wondrack et al. (1996)
Virulence genes	*asa1-F* *asa1-R*	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	375	Denaturation 95 °C 180 s, 30 cycles at 95 °C 30 s, 56 °C 30 s, 72 °C 60 s, 72 °C 600 s	Vankerckhoven et al. (2004)
	cylA-F *cylA-R*	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688		
	esp-F *esp-R*	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	510		
	gelE-F *gelE-R*	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	213		
	hyl-F *hyl-R*	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	276		

Vankerckhoven et al. (2004). In this case, 0.2 μM of primers *asa1* and *gelE* each and 0.4 μM of primers *cylA*, *esp.* and *hyl* each were used with PCR cycling conditions described in Table 1.

Electrophoresis and sequencing

The success of all PCR amplifications was determined by electrophoresis (Jordaan and Bezuidenhout, 2013). PCR products of efflux pump and virulence genes were purified as described by Li et al. (2010) as well as using the ZR DNA Sequencing Clean-up Kit (Zymo Research, USA). DNA sequencing was done using the BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, UK) on an ABI 3130 Genetic Analyser (Applied Biosystems-Hitachi).

All sequences obtained were compared to published gene sequences in the National Center of Biotechnology Information Database (NCBI), GenBank, using BLASTN to determine their identity. Representative bacterial nucleotide efflux sequences were submitted to the Genbank database under accession numbers: KU182369-KU182389.

Results

Five surface water systems in the NWP, South Africa, were screened for the presence of *Enterococcus* spp. Presented in Table 2 are the virulence and efflux pump gene trends as well as antibiotic resistance patterns of the *Enterococcus* spp. *Enterococcus* isolates from the Mooi and Vaal River harboured four of the five virulence genes screened. Whereas, *Enterococcus* spp. from the Upper Harts, Lower Harts and Vaal River harboured two of the five virulence factors of interest.

Presence of virulence genes in *Enterococcus* spp. isolated

Aggregation substance (*asa1)* was most frequently detected among the enterococci from Barberspan. Nonetheless, *asa1* was also present in isolates from the Upper Harts and individual isolates from the Mooi and Schoonspruit Rivers. Furthermore, cytolysin (*cylA*) was frequently observed in *Enterococcus* spp. isolated from the Mooi, Schoonspruit and Vaal Rivers. Gelatinase (*gelE*) was harboured predominantly by isolates from the Upper Harts River. However, this virulence gene was also prevalent in enterococci isolates from

Table 2 Virulence and efflux pump gene trends as well as antibiotic resistance patterns of *Enterococcus* spp. isolated from five surface water systems, in the NWP, South Africa

Water system	No. of sites	No. of *Enterococcus* spp.	*asa1* n (%)	*cylA* n (%)	*gelE* n (%)	*esp n (%)*	*hyl* n(%)	*msrC* n (%)	*tetL* n (%)
Upper Harts River	7	34	5 (15)	0	20 (59)	0	0	1 (3)	0
Barberspan	4	18	8 (44)	5 (28)	6 (33)	0	0	1 (6)	3 (17)
Lower Harts River	6	20	0	3 (15)	0	0	2 (10)	1 (15)	0
Mooi River	6	13	1 (8)	6 (46)	4 (31)	0	2 (15)	4 (31)	4 (31)
Schoonspruit River	4	17	1 (6)	9 (53)	6 (35)	0	3 (18)	1 (6)	6 (35)
Vaal River	5	22	0	10 (46)	2 (9)	0	0	1 (5)	2 (9)

Percentage (%) was determined as a function of the number of isolates of the specific *Enterococcus* spp. thus (n/(N) × 100)

Barberspan, Mooi and Schoonspruit Rivers. In addition, hyaluronidase (*hyl*) was not detected in high levels, yet, it was observed in *Enterococcus* spp. isolated from the Lower Harts, Mooi River and Schoonspruit Rivers. The presence of enterococcal surface protein (*esp*) was investigated and this virulence gene was not detected in any of the isolates.

Presence of efflux pump genes in the screened *Enterococcus* spp. and their multiple antibiotic resistance patterns

Efflux pump genes for *msrC* and *tetL* were detected in some of the isolates (Table 2). The *msrC* efflux pump gene was present among isolates from all the water systems. On the other hand, the *tetL* efflux pump gene was predominant in *Enterococcus* spp. isolated from the Schoonspruit and Mooi Rivers. It was also detected among isolates from the Vaal River and Barberspan. The *mefA* and *tetK* efflux pump genes were not detected in any of the screened *Enterococcus* isolates.

The antibiotic MAR phenotypes of isolates across the five surface water systems of interest were determined. Resistance of *Enterococcus* spp. to β-lactam, fluoroquinolones and vancomycin was common across all surface water systems. However, some β-lactam resistant isolates were susceptible to one or more of the other β-lactam antibiotics. Furthermore, the highest MAR diversity patterns were observed in the Lower Harts River.

The overall antimicrobial resistance of the 124 isolates was determined, and results are expressed as percentages of isolates that were resistant to the various antibiotics (Table 3). Most of these isolates were resistant to penicillin (70 %) and vancomycin (69 %). Between 40 and 55 % were resistant to ampicillin, amoxicillin, erythromycin, neomycin, tetracycline and ciprofloxacin. Lower *Enterococcus* numbers were also resistant to chloramphenicol (19 %) and streptomycin (6 %).

When considering the antibiotic resistance patterns of various species results, in Table 3, it is demonstrated that a high percentage of *E. faecalis* spp. was resistant to β-lactam antibiotics: ampicillin, amoxicillin and penicillin as well as the fluoroquinolone and ciprofloxacin. Furthermore, a high percentage of this species was resistant to vancomycin and to a lesser extent to neomycin, erythromycin and tetracycline. Although the exact percentages are different, similar trends

Table 3 Antibiotic resistant *Enterococcus* spp. isolated from surface water samples

Species	No. of isolates	Antibiotics									
	(N)	AMP n (%)[a]	AMOX n (%)[a]	PEN n (%)[a]	NE n (%)[a]	STR n (%)[a]	VAN n (%)[a]	CHL n (%)[a]	CIP n (%)[a]	ERY n (%)[a]	TET n (%)[a]
E. faecium	30	11 (36)	15 (50)	20 (66)	13 (43)	3 (10)	19 (63)	6 (20)	14 (47)	22 (73)	14 (46)
E. faecalis	37	23 (62)	24 (64)	30 (81)	21 (57)	2 (5)	27 (73)	6 (6)	9 (24)	18 (48)	18 (48)
E. mundtii	36	17 (47)	8 (22)	26 (72)	21 (58)	1 (3)	23 (64)	6 (17)	21 (58)	16 (44)	20 (55)
E. casseliflavus	14	5 (35)	4 (29)	8 (57)	7 (50)	1 (7)	11 (78)	3 (21)	1 (7)	8 (57)	4 (28)
E. gallinarum	5	2 (40)	1 (20)	2 (40)	2 (40)	0	5 (100)	1 (20)	0	3 (60)	2 (40)
E. hirae	1	0	0	0	1 (100)	0	0	0	1 (100)	0	0
E. sulfureus	1	1 (100)	1 (100)	1 (100)	0	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Total	124	59	53	87	65	8	86	23	47	68	59

AMP Ampicillin (10 μg), *AMOX* Amoxicillin (10 μg), *PEN* Penicillin G (10 μg), *NE* Neomycin (30 μg), *STR* Streptomycin (300 μg), *VAN* Vancomycin (30 μg), *CHL* Chloramphenicol (30 μg), *CIP* Ciprofloxacin (5 μg), *ERY* Erythromycin (15 μg), *TET* Tetracycline (30 μg)

[a] Percentage (%) was determined as a function of the number of isolates of the specific *Enterococcus* spp. thus (n/(N) × 100).

of *E. faecium* and *E. mundtii* isolates were resistant to the antibiotics listed above. Low percentages of the various *Enterococcus* spp. were resistant to streptomycin and chloramphenicol.

Of the 124 *Enterococcus* isolates screened, 22 (18 %) isolates harboured at least one efflux pump gene (Table 4). While among these, 4 (18 %) harboured two efflux pump genes. The antibiotic efflux pump genes were predominantly detected in *Enterococcus faecium* (33 %, 10/30) followed by *E. casseliflavus* (29 %, 4/14), *E. mundtii* (14 %, 5/36) and *E. faecalis* (8 %, 3/37) spp. In contrast, no antibiotic efflux pump genes were detected in *E. gallinarum*, *E. hirae* and *E. sulfureus* spp.

Virulence and efflux pump gene trends as well as antibiotic resistance patterns

All the *Enterococcus* spp. isolated were screened for the presence of selected efflux pump and virulence genes. In Table 4, the genotypic characteristics of virulence genes and phenotypic characteristics of 22 antimicrobial resistant *Enterococcus* spp. that carried efflux pump genes are provided. Four of the predominantly isolated *Enterococcus* spp. tested positive for efflux pump genes. Individual strains of *E. faecium*, *E. faecalis* and *E. mundtii* were resistant to β-lactam antibiotics and at least one antibiotic from another class. Eighty-six percent (19/22) of the MAR phenotypes observed comprised tetracycline, while 55 % (19/22) and 45 % (10/22) comprised ciprofloxacin and erythromycin. The highest antibiotic resistance phenotypes were observed in *E. faecium* spp. with three isolates being resistant to seven of the ten screened antibiotics. However, one *E. mundtti* isolate also displayed resistance to seven antibiotics.

The *tetL* efflux gene was the most frequently determined gene among the 59 tetracycline resistant *Enterococcus* spp. and was found in 17 (28 %) isolates. However, the *tetK* efflux gene was not detected. Furthermore, the *msrC* efflux gene was detected in 9 (13 %) of the erythromycin resistant isolates. However, none of the *Enterococcus* spp. harboured the *mefA* efflux gene, despite 68 of them showing resistance to erythromycin.

Additionally, the presence of virulence factors in the *Enterococcus* spp. harbouring resistance genes was also determined. As depicted in Table 4, of the *Enterococcus* spp.

Table 4 Genotypic and phenotypic characteristics of *Enterococcus* spp. that carried efflux pump genes

Species	Antibiotic resistance phenotype	Efflux pump genes	Virulence factors
E. faecium			
SR12	AMP, AMOX, PG, VAN, CIP, ERY, TET	*msrC*, *tetL*	*cylA*
SR4	AMP, AMOX, PG, VAN, CIP, ERY, TET	*tetL*	*cylA*
VR5	AMP, PG, VAN, CHLOR, CIP, ERY, TET	*msrC*	*cylA*
MR2	AMOX, PG, VAN, CIP, ERY	*msrC*	*cylA*, *hyl*
MR6	AMOX, PG, CIP, ERY, TET	*msrC*, *tetL*	*cylA*, *hyl*
MR13	AMOX, ERY, TET	*msrC*	None detected
MR18	AMOX, CIP, ERY	*msrC*	*cylA*
MR14	AMOX, CIP	*tetL*	*cylA*
LHR6	AMOX, NE, VAN, CIP, TET	*tetL*	*gelE*, *hyl*
UHR37	PG, CIP, ERY, TET	*msrC*	*asa1*
E. faecalis			
VR8	AMOX, PG, NE, VAN, ER, TET	*tetL*	*asa1*, *gelE*
VR10	AMP, AMOX, PG, NE, TET	*tetL*	*gel*
MR11	AMOX, VAN, CIP, ERY, TET	*msrC*, *tetL*	*cylA*
E. casseliflavus			
SR9	NE, VAN, TET	*tetL*	*cylA*
VR2	NE, VAN, ER, TET	*tetL*	*cylA*
VR3	PG, VAN, TET	*tetL*	*cylA*
MR9	CIP, ERY, TET	*msrC*, *tetL*	*asa1*, *gelE*
E. mundtii			
B1	PG, NE, VAN, OT, TET	*tetL*	*cylA*
B4	AMP, AMOX, PG, NE, TET	*tetL*	*cylA*
B9	PG, TET	*tetL*	*asa1*
LHR3	AMP, AMOX, PG, NE, VAN, CHLOR, TET	*tetL*	None detected
LHR12	PG, NE, TET	*tetL*	None detected

harbouring efflux pump genes also harboured one (14; 63 %) or two (5; 23 %) virulence factors. Cytolysin (*cylA*) was the most predominant virulence gene detected in 13 (59 %) of *Enterococcus* spp. harbouring efflux pump genes. The virulence genes *asa1* (18 %), *gelE* (14 %) and *hyl* (14 %) were also detected. *Enterococcus faecium* spp. harboured the most virulence genes. No virulence genes were detected in 3 (14 %) of the isolates harbouring efflux pump genes.

Discussion

To date, research on efflux pump systems and virulence in enterococci have focused mainly on clinical isolates with the assumption that these mediate higher public health threats (Dada et al. 2013). However, recent reports have illustrated that isolates obtained from municipal sewerage polluted environmental sources could directly pose threats to the health of users (Iweriebor et al. 2015). Such sources may permit the dissemination of antibiotic resistance and virulent bacteria (Carvalho et al. 2014). Several studies around the world have reported the presence of antibiotic resistant genes in *Enterococcus* spp. isolated from water systems (Schwartz et al. 2003; Santiago-Rodriguez et al. 2013). However, little is known about the genotype of antibiotic resistant and virulence genes and their distribution among enterococci isolated from South African waters. Furthermore, a number of reports, globally, affirm that enterococci with the highest virulence are of clinical origin, followed by industrial isolates (Fisher and Phillips 2009). However, notably missing are the environmental strains. In this study, the presence of antibiotic resistance and virulence genes in *Enterococcus* spp. isolated from surface water systems was determined.

Virulence and efflux pump gene trends as well as antibiotic resistance patterns

Virulence genes were mostly prevalent in *Enterococcus* spp. of the Mooi River and Vaal River. These two water systems support a variety of urban centres, agricultural and recreational activities (DWAF 2004; DWAF 2009). The recreational activities supported include swimming, fishing and angling (Pantshwa et al. 2009). Thus, the presence of these virulence genes in these river systems could allow for the dissemination of virulence genes from the environment to humans through open wounds and other routes. Furthermore, efflux pump genes were identified in all five surface water systems. The *tetL* gene was, however, present in the surface water systems screened with the exception of the Harts River. To our knowledge, this is the first report illustrating the presence of *Enterococcus* spp. harbouring efflux pump genes in the five surface water systems of interest. Furthermore, several of the *Enterococcus* spp. isolated were resistant to β-lactam, fluoroquinolone and vancomycin antibiotics. This is not surprising seeing that the fluoroquinolone antibiotic ciprofloxacin is regularly prescribed in South Africa to females between ages of 12 and 18 years to treat urinary tract infections (Agyakwa 2014). Furthermore, increased intrinsic resistance to β-lactams has been reported in clinical *Enterococcus* spp. as a result of their penicillin-binding proteins (Chen and Zervos 2009; Hollenbeck and Rice 2012). Also, the presence of vancomycin resistant bacteria in South African clinical enterococcus isolates has previously been reported (Budavari et al. 1997; McCarthy et al. 2000; Iweriebor et al. 2015). The presence of vancomycin resistant enterococci is, however, a cause for concern because of their ability to transfer the vancomycin resistance determinant *van* to other bacterial species posing a public health threat (Iweriebor et al. 2015).

According to Chen and Zervos (2009), cell-wall inhibitors such as penicillin, ampicillin and vancomycin alongside aminoglycosides are used for the treatment of serious enterococcal infections. Thus, the observed multiple antibiotic resistance of *Enterococcus* spp. screened in this study is worrisome as it relates to the potential therapeutic failure when antibiotics from several classes are used to attain synergistic bactericidal activity (Chen and Zervos 2009).

Previous studies have also reported the resistance of *Enterococcus* spp. to fluoroquinolones in water and wastewater samples (Martins da Costa et al. 2006; Moore et al. 2008). Carvalho et al. (2014), ascribed resistance of vancomycin resistant *Enterococcus* isolates present in marine ecosystems to be the result of faecal pollution. Resistance to erythromycin, predominantly found among *E. faecium* spp., was also observed in *E. faecalis*, *E. gallinarum* and *E. casseliflavus.* This is in accordance with findings of Łuczkiewicz et al. (2010). The observed resistance of *Enterococcus* spp. to erythromycin can be a result of its extensive use in livestock breeding programmes and treatment of infections where resistance or hypersensitivity to penicillin is suspected (Blanch et al. 2003; Duarte et al. 2005).

According to Łuczkiewicz et al. (2010), the presence of fluoroquinolones in MAR phenotypes of gram-positive organisms is an increasing problem. Furthermore, the presence and association of ERY-TET in the MAR phenotypes supports suggestions of a co-selection mechanism between erythromycin and tetracycline resistant organisms (Martins da Costa et al. 2006). Luczkiewicz et al. (2010) suggested that resistance to erythromycin could influence and raise resistance to tetracycline. Considering the promiscuity of *Enterococcus* spp., this latter suggestion is not an abnormal phenomenon as macrolides and tetracyclines are commonly and extensively used in clinical and animal health therapy, allowing for the transferal of bacteria resistant to such antibiotics into the environment (Blanch et al. 2003; da Silva et al. 2006).

Multiple antibiotic resistance phenotypes observed among the *Enterococcus* isolates that also harbour antibiotic

resistance efflux pump and virulence genes respectively is a cause for concern. The water systems that were sampled are used for various purposes in which direct exposure is common. Such water may pose a health threat to the individuals especially the immuno-compromised.

Presence of efflux pump genes

Of the four (*mefA*, *msrC*, *tetL* and *tetK*) efflux pump genes screened, *mefA* and *tetK* genes were not detected. The absence of these two efflux pump genes in *Enterococcus* spp. has been reported previously (López et al. 2010; Portillo et al. 2000; Roberts et al. 1999). In the case of erythromycin resistance, it could be that different mechanism or efflux pumps could be conferring the resistance phenotype (Chouchani et al., 2012). However, Portillio et al. (2000) has reported the occurrence of *mefA* genes in *Enterococcus* spp. Thus, including these genes when testing for erythromycin resistance determinant are justified.

In this study, *tetL* was the main genetic determinant associated with the resistance to tetracycline. This is similar to the study of Valenzuela et al. (2013) that also found that *tetL* genes were the most predominant among tetracycline resistant enterococci. However, in the present study, this gene as well as *tetK* could not be detected in some of the tetracycline resistant isolates. In these cases, other tetracycline resistance determinants (e.g. *tetM*, *tetO*, *tetQ* or *tetS*) could have been responsible for the observed phenotype. However, detecting *tetL* efflux pump genes in several *Enterococcus* spp. in this study is significant because few studies have focused on the prevalence of tetracycline resistance genes in recreational waters (Santiago-Rodriguez et al. 2013).

The *msrC* gene was detected in some of the erythromycin resistant *Enterococcus* spp. screened. This gene, *msrC* , codes for an efflux pump that confers low resistance against macrolides and type B streptogramins (Singh et al. 2001). The *msrC* gene was predominantly harboured by *E. faecium* isolates. Portillo et al. (2000) also reported that the *msrC* gene is distributed among erythromycin resistant *E. faecium*. These authors suggested that this gene is species specific. However, in the present study, *msrC* was also detected in one *E. faecalis* and one *E. casselflavus* isolate. This finding is supported by previous studies that reported the presence of *msrC* genes conferring resistance to erythromycin in *E. faecalis*, *E. durans*, *E. lactis* and *E. casseliflavus* spp. (Aakra et al. 2005; Thumu and Halami 2012). Thumu and Halami (2012) further advocated that the presence of *msrC* in different *Enterococcus* spp. could be a result of horizontal gene transfer. However, sequencing regions surrounding this gene would be mandatory in order to reveal the presence of elements that would suggest the potential mobility of this gene.

Presence of virulence determinants

Virulence genes were detected among *E. faecium*, *E. faecalis*, *E. casselfilavus* and *E. mundtii* spp. This finding is similar to that of Dada et al. (2013), Iwerebor et al. (2015) and Sidhu et al. (2014). The cytolysin determinant (*cylA*) was detected most frequently. This virulence factor is of interest due to its ability to enhance enterococcal virulence (Vankerckhoven et al. 2004). Furthermore, few of the MAR enterococci also carried the virulence genes *asa1*, *gelE* and *hyl*. The virulence factors, *asa1* and *gelE*, are involved in bacterial adhesion and the catabolism of various molecules such as gelatin, collagen and fibrinogen (Vankerckhoven et al. 2004). Furthermore, the low levels of virulence genes in surface water systems is in agreement to the findings of a study recently performed in South Africa (Iweriebor et al. 2015). Nonetheless, the presence of virulence genes in *Enterococcus* spp. isolated from surface water sources is a cause for concern. These virulence factors permit colonization and invasion of a host's tissue as well as translocation through epithelial cells in order to evade the host's immune response (Vu and Carvhalo 2011). Therefore, their presence in *Enterococcus* spp. isolated from surface water sources used for various agricultural and recreational activities poses a health risk, particularly for the immune-compromised sector of the population.

Motivation for use as an additional indicator of *Enterococcus* in water quality assessments.

Conclusions

In the current study, the antimicrobial susceptibility patterns of *Enterococcus* spp. isolated from various surface water systems in the North West Province, South Africa were determined. Many of these enterococci were resistant to multiple antibiotics and resistance to tetracycline and erythromycin may be linked to efflux pump genes. A large proportion of the MAR *Enterococci* spp. also tested positive for virulence genes. The presence of efflux pumps conferring resistance to multiple antibiotics is a cause for concern since efflux pumps are known for their remarkable ubiquitous nature and that they a have broad substrate range. The expression and up regulation of these efflux pump genes is particularly important as they could be contributing to multiple antibiotic resistance of the potential pathogenic bacteria. This is a cause for concern as these isolates could be a public health risk particularly for immune-compromised individuals. The potential source of these isolates needs to be investigated.

Acknowledgments The authors wish to thank Dr. C Mienie for assistance with sequencing, Mr Gustav Havenga for the map, the National Research Foundation Deutscher Akademischer Austauchdienst (NRF-

DAAD) for a bursary to LGM and Water Research Commission of South Africa (K5/1966 & K5/2347) for financial support. The views expressed are those of the authors and not of the funding agencies.

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