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# Molecular detection of antibiotic resistance and virulence gene determinants of *Enterococcus* species isolated from coastal water in the Eastern Cape Province, South Africa

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## ABSTRACT

Enterococci are a group of significant disease-causing bacteria, which have developed resistance to several conventionally used antibiotics. Of the 67 confirmed *Enterococcus* species from Kidd's Beach, 40 (59.7%) were *E. faecium*, 19 (28.4%) were *E. faecalis*. The highest level of resistance was observed against rifampicin (83.6%) followed by erythromycin (64.2%), tetracycline (52.2%), linezolid (46.3%), tetracycline (46.3%), and vancomycin (32.8%). Ninety-six per cent of the *Enterococcus* spp. was found to be multi-drug resistant. MAR indices vary from 0.3 to 0.9. Two virulence determinants (*ace* and *gelE*) were detected and six resistance determinants were identified as follows: *ermB* (19%), *tetM* (30%), *tetL* (19%), *gyrA* (13%), *ampC* (1.5%) and *Van C2/3* (4.5%). The presence of enterococci (*E. faecium* and *E. faecalis*) in Kidd's Beach waters harbouring virulence genes that facilitate multiple antibiotic resistance signifies a possible health threat for beach goers.

## KEYWORDS

*Enterococcus* spp; antibiotic resistance; multiple antibiotic resistance; virulence factor; health threat

## Introduction

Antibiotic-resistant bacteria have been detected in several environmental sources comprising untreated and treated wastewater [1,2], rivers [3] and coastal water [4,5]. The incidence of multidrug-resistant bacteria has been increasing at an alarming rate [6–11], causing high rates of treatment failure [12,13], increased incidence and severity of infections [14,15], and ultimately, increased mortality [16]. If unabated, it could instigate a 'post-antibiotic era', where infectious diseases could become incurable [17]. There would then be serious socio-economic consequences for families and world economies. Patients would have to be hospitalised for longer periods at a time leading to loss of working hours, and there would be the loss of skilled labour to premature deaths [18].

Enterococci species are among pathogens known to be intrinsically resistant to a wide range of antibiotics including beta-lactams, aminoglycosides and ampicillins [19,20].

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This bacterium is one of the microflora inhabiting the gastrointestinal tract of warm-blooded animals, including human beings [21]. The presence of enterococci in the aquatic environment therefore indicates faecal contamination [22,23]. Although microbial pollution of waterbodies can be linked to diffuse sources of faecal pollution including livestock, wildlife and municipal wastewater effluents [24], the presence of antibiotic-resistant (AR) enterococci in marine coastal environments has been linked to human faecal pollution [25,26]. Vallero *et al.* [27] reported that over 90% of bacterial strains recovered from coastal water are resistant to at least three antibiotics, and about 20% are resilient to at least five antibiotics.

Waterborne *Enterococcus* species have attracted particular clinical interest because they are labelled as opportunistic pathogens that cause nosocomial infections [28–30]. Reports by Fisher and Phillips [31] and Mendes *et al.* [32] have shown that among *Enterococcus* species, *E. faecium* and *E. faecalis* are responsible for high levels of infection in human beings and animals, causing bacteraemia, endocarditis and pyelonephritis. Part of this observed virulence and antimicrobial resistance has been attributed to the vulnerability of enterococci species to acquire transposomes carrying varying genetic elements [33]. Pathogenic *Enterococcus* species also aid the pathogenicity of other bacteria through interspecies conjugation of genetic elements required for colonisation of the host, breaching of host defence mechanisms and production of toxins for induction of inflammation [34,35]. In enterococci, the presence of genes encoding virulence factors including enterococcal aggregation substance (*asa1*), cytolysin (*cylA*), surface protein (*esp*), collagen-binding protein (*ace*), endocarditis antigen (*efaA*), hyaluronidase (*hyl*) and gelatinase (*gelE*) has been determined [36,37]. Although the virulence and antimicrobial resistance of *Enterococcus* species has been reported in many settings, there remains an information gap on the occurrence, virulence and antimicrobial resistance patterns of these species in recreational beaches of several countries. Thus, the aim of this research was to evaluate the virulence factors and antimicrobial susceptibility patterns of *Enterococcus* species recovered from the coastal waters of a resort beach in the Eastern Cape Province of South Africa.

## Materials and methods

### *Description of study area and collection of water samples*

Six sampling points were identified along Kidd's Beach which is located in Buffalo City Metropolitan Municipality in the Eastern Cape Province of South Africa. Sampling points were coded as follows: KP1, KP2 and KP3 (all beach water); KP4 and KP5 (canal water), and KP6, pool water (Figure 1). Presumed causes of faecal contamination affecting the sampling points included river discharge, coastal birds, swimming, surfing, animal inputs, and municipal sewage discharge. The sewage discharges were more pronounced at KP3 and KP6. Water samples were collected monthly in sterile 1 L bottles at each of the six sites between November 2017 and April 2018 and transported to laboratory in cooler boxes containing ice and processed within 6 h of collection.

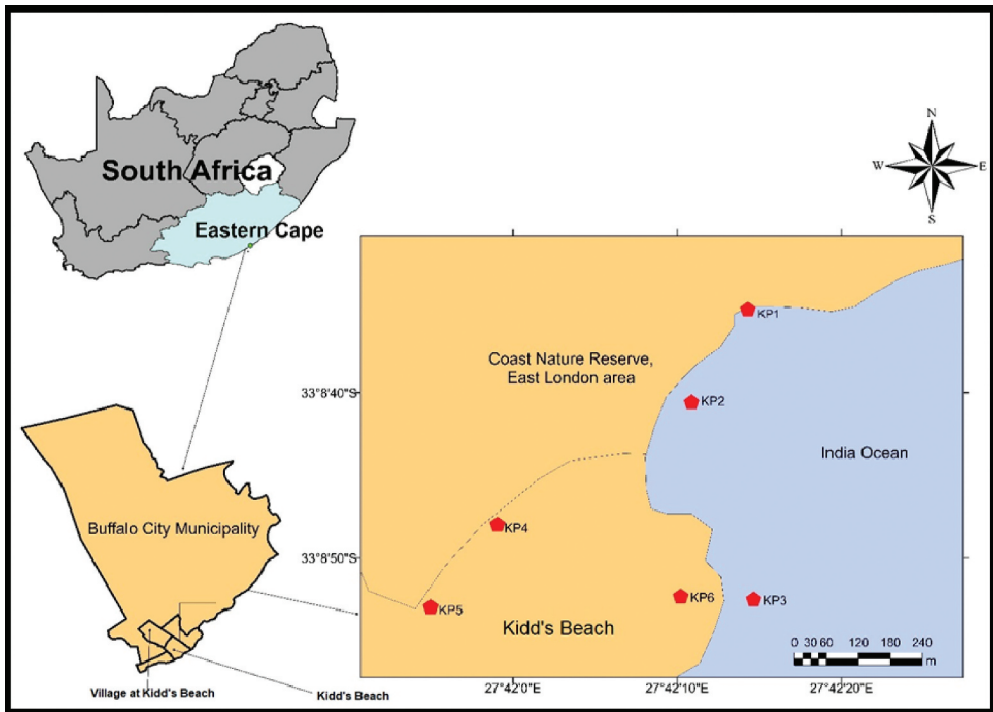


Figure 1. Map of Kidd's beach.

### *Isolation of presumptive Enterococcus colonies*

Water samples were subjected to 10-fold serial dilution after which 100 mL aliquots of each dilution were filtered using nitrocellulose membrane filters (0.45- $\mu\text{m}$  pore size, Millipore, Ireland) following the membrane filtration technique for the isolation and enumeration of faecal coliforms according to standard methods for microbiological water analysis [38]. The filters were aseptically transferred grid-up onto Bile Aesculin Azide agar and gently pressed to be totally in touch with the media surface and incubated at 37°C for 48 h. Following incubation, a total of 409 presumptive *Enterococcus* colonies presenting a black to dark brown colouration were purified by sub-culturing onto nutrient agar till axenic cultures were obtained. Lone standing colonies from the axenic culture plates were transferred onto 25% glycerol and stored at 4°C till further analysis.

### *DNA extraction and PCR confirmation of presumptive Enterococcus isolates*

Total genomic DNA was extracted using the boiling method as described by Magueri *et al.* [39]. Confirmative PCR identification of enterococci was done based on the method described by Ke *et al.* [40] and Adeniji *et al.* [41] using genus-specific primers targeting the *tuf*-gene using *E. faecalis* (DSM 20,478) as a positive control.

**Table 1.** Virulence genes primers.

Gene	PCR primer sequence (5'–3')	Amplicon size (bp)	Reference
<i>Hyl</i> <b>HYLn1</b>	ACAGAAGAGCTGCAGGAAATG	276	Vankerckhoven <i>et al.</i> [43]
<i>Hyl</i> <b>HYL n2</b>	-GACTGACGTCCAAGTTTCCAA		
<i>cylA</i> <b>CYT I</b>	ACTCGGGGATTGATAGGC	688	Vankerckhoven <i>et al.</i> [43]
<b>CYT II</b>	GCTGCTAAAGCTGCGCTT		
<i>esp</i> <b>ESP46</b>	TTACCAAGATGGTTCTGTAGGCAC	913	Vankerckhoven <i>et al.</i> [43]
<b>ESP47</b>	CCAAGTATACTTAGCATCTTTTGG		
<i>efaA</i> <b>efaA1</b>	CGTGAGAAAGAAATGGAGGA	499	Mannu <i>et al.</i> [42]
<b>efaA2</b>	CTACTAACACGTCACGAATG		
<i>Ace</i> <b>ACE1</b>	AAAGTAGAATTAGATCCACAC	320	Mannu <i>et al.</i> [42]
<b>ACE2</b>	TCTATCACATTTCGGTTGCG		
<i>gelE</i> <b>gelE1</b>	AGTTCATGTCTATTTTCTTCAC	402	Mannu <i>et al.</i> [42]
<b>gelE2</b>	CTTCATTATTTTACACGTTTG		

### Screening for virulence genes

PCR confirmed enterococci strains were screened for six virulence genes formerly described by Mannu *et al.* [42] and Vankerckhoven *et al.* [43] using gene-specific primer sets listed in Table 1. Multiplex PCR was used with the following cycling conditions: denaturation at 94°C/3 min; 35 cycles of denaturation at 93°C/60 s, annealing at 50°C/60 s, elongation at 72°C/60 s, and a final extension step at 72°C for 10 min. The products were resolved in 2% agarose gel and visualised under an Ultraviolet (UV) transilluminator as already described.

### Antibiotic susceptibility testing

Antimicrobial susceptibility testing was carried out using disk diffusion method on Muller Hinton agar (MHA) following the method of Kirby-Bauer according to the recommendation of CLSI [44]. Four to five (4–5) colonies at log phase were picked with a sterile inoculating loop, transferred into test tubes containing 5 mL of sterile 0.85% normal saline and lightly vortexed. The bacterial suspensions were adjusted to 0.5 McFarland standards as described by Panda *et al.* [45]. Mueller Hinton agar plates were inoculated by uniformly spread plating 100 µl of the bacterial suspensions over the surface of the agar, and allowing the plates to dry before antibiotic discs were placed on them. Each *Enterococcus* isolate was tested against a panel of 11 antibiotics (Mast Diagnostics, UK) including ampicillin (AP, 10 µg), vancomycin (VA, 30 µg), linezolid (LZD, 30 µg), ciprofloxacin (CIP, 5 µg), levofloxacin (LEV, 5 µg), norfloxacin (NOR, 10 µg), nitrofurantoin (NIT, 300 µg), chloramphenicol (C, 30 µg), tetracycline (T, 30 µg), erythromycin (E, 15 µg) and rifampicin (RP, 5 µg). Subsequently, the plates were incubated at 37°C for 24 h and zones of inhibition were measured and classified as either susceptible (S), intermediate (I), and resistant (R), using the CLSI [43] chart for interpretation.

### Determination of multiple antibiotic-resistant phenotypes (MARPs)

Following the method of Wose *et al.* [46], MARPs were generated for each isolate and sample site. The multiple antibiotic resistance index (MARI) for each isolate was done using the mathematical expression of Blasco *et al.* [47] as follows:

$$(MAR) \text{ index} = c/d$$

where  $c$  is the number of antibiotics that the strain was resistant against and  $d$  is the aggregate number of antibiotics against which the individual strains was tested.

The antibiotic resistance index (ARI) was calculated for each sampling site using the formula described by Kuperman [48], mathematically expressed as:

$$ARI = A/B(Z),$$

where  $A$  is the total antibiotic resistance score of all strains from the sample,  $B$  is the number of antibiotics and  $Z$  is the number of strains from the sample. Thereafter, the prevalence of relevant antibiotic resistance genes was determined using PCR.

### Detection of antibiotic-resistant genes

The occurrence of antibiotic resistance genes among those that showed resistance to the antibiotics was determined using gene-specific primers synthesised by Inqaba Biotech (Pretoria, South Africa). PCR amplification was carried out in 25  $\mu\text{L}$  reaction mixtures consisting of 12.5  $\mu\text{L}$  of PCR master mix (Thermo Scientific, (EU) Lithuania), 0.5  $\mu\text{L}$  of each primer (Inqaba Biotech, SA), 5  $\mu\text{L}$  of template DNA and 6.5  $\mu\text{L}$  of nuclease-free water. The primer sets, targeted genes, PCR conditions, antibiotic family and base pairs are all listed in Table 2.

### Data analysis

IBM SPSS Statistics version 25 was employed for data analysis in this work, and the significant levels were set at  $p < 0.05$ . Significance of difference in resistance among the isolates with respect to different sampling locations was determined by one-way analysis of variance (ANOVA).

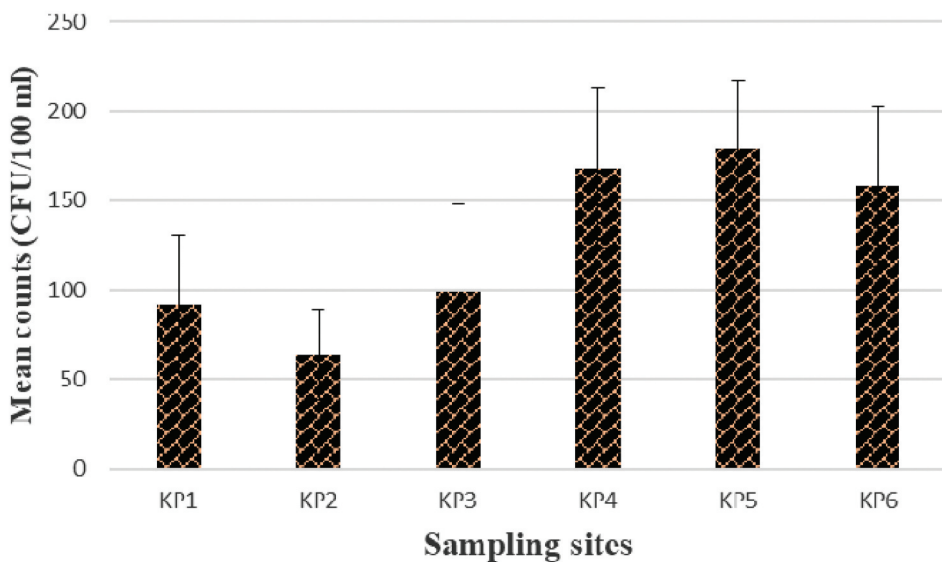
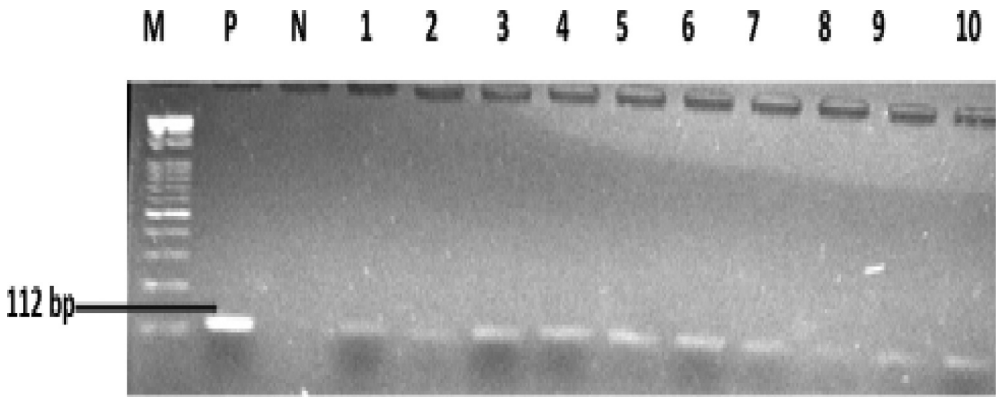


Figure 2. Presumptive *Enterococcus* counts obtained from each sampling site.



**Figure 3.** PCR products of the amplification of *tuf*-gene. M = Molecular ladder (100 bp), lane 1–10 positive isolates. Lane N; negative control.

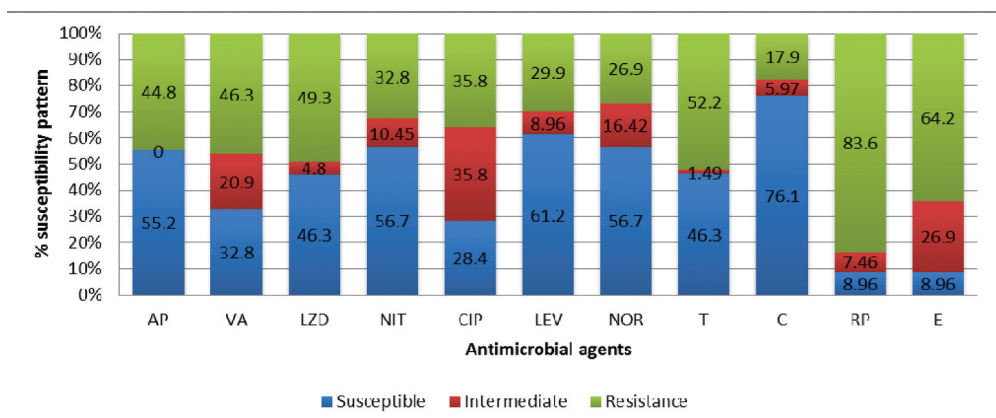
## Results

### PCR confirmation and occurrence of *Enterococcus* isolates from six sampling sites

Presumptive *Enterococcus* species were identified in counts ranging from 64 to 168 CFU/100 mL of water samples at the six sampling sites. In total, 67 isolates were obtained from the Kidd’s Beach coastal waters after PCR confirmation of the isolates. PCR speciation of the isolates showed that 19 (28.4%) were *Enterococcus faecalis* and 40 (59.7%) were *Enterococcus faecium*. **Figure 2** shows the presumptive enterococci counts obtained at each sampling site. **Figure 3** shows the gel electrophoresis picture for the PCR amplification of *tuf* gene in the *Enterococcus* species.

### Phenotypic pattern of resistance to antibiotics

High levels of resistance were detected against rifampicin (83.6%), erythromycin (64.2%) and tetracycline (52.2%). The isolates also showed resistance to other

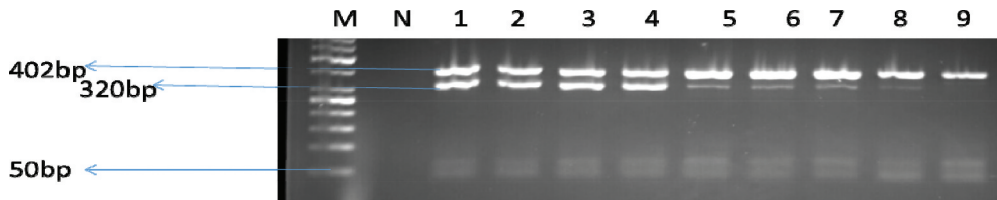
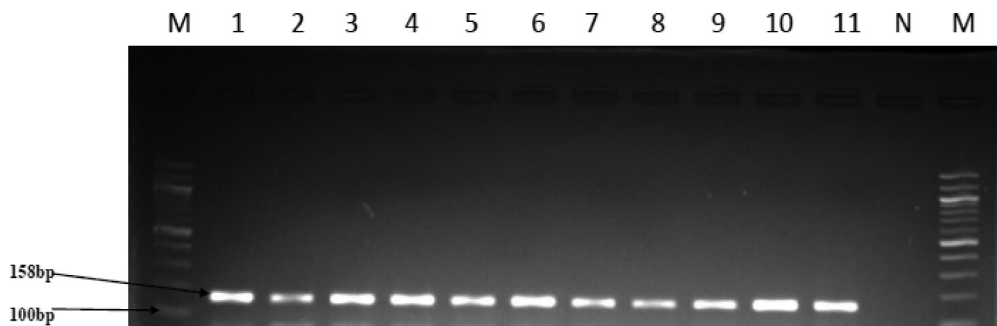


**Figure 4.** Susceptibility pattern of *Enterococcus* isolates against selected antimicrobial agents.



**Table 4.** Frequency of virulence genes among *E. faecalis* and *E. faecium* isolated from coastal water.

Species (n = 67)	Ace (320 bp)	gelE (402 bp)
<i>E. faecalis</i>	9 (13.4%)	10 (15%)
<i>E. faecium</i>	1 (1.5%)	2 (3%)
Total	10	12

**Figure 5.** A gel electrophoresis showing the distribution of virulence genes among *E. faecalis* and *E. faecium*. Lane M: Molecular Marker (50 bp); Lane N: Negative control; Lane 1–9 positive isolates.**Figure 6.** A gel electrophoresis showing the tetracycline resistance tetM gene. M = Molecular ladder (100 bp), lane 1–11 positive isolates. Lane N; negative control, M: Molecular ladder.**Table 5.** Frequency of resistance genes detected among the enterococcus isolates.

Class	Antibiotic	Resistance Gene	No. of Strain
Glycopeptides	Vancomycin	<i>van C2/3</i>	3 (4.5%)
Macrolides	Erythromycin	<i>ermB</i>	6 (19%)
beta-lactams	Ampicillin	<i>ampC</i>	1 (1.5%)
Flouroquinnone	Ciprofloxacin	<i>gyrA</i>	9 (13%)
Tetracycline	Tetracycline	<i>tet L</i>	13 (19%)
		<i>tet M</i>	20 (30%)

antibiotics as follows: vancomycin (46.3%), linezolid (49.3%), ampicillin (44.8%) and ciprofloxacin (35.8%). **Figure 4** shows the full spectrum of the observed phenotypic resistance and susceptibility patterns against the tested antibiotics.



AP- Ampicillin 10 µg, VA- Vancomycin, 30 µg, LZD – Linezolid 30 µg, CIP – ciprofloxacin 5 µg, LEV- levofloxacin 5 µg, NOR- Norfloxacin 10 µg, NIT- nitrofurantoin 300 µg C – chloramphenicol 30 µg, T- tetracycline 30 µg, E – erythromycin 15 µg and RP- rifampicin 5 µg.

Table 3 shows the antimicrobials, resistance patterns and number observed across different sampling locations with respect to the prevalence of multiple antibiotic resistance phenotypes (MARPs) of the *Enterococcus* isolates.

### Identification of virulence genes

The 67 confirmed enterococci isolates were further analysed for the presence of the targeted virulence genes *gelE*, and *ace* gene. A total of 11 strains were positive for the *ace* gene and 13 for the *gelE* gene (Table 4). The gel showing the PCR amplification product of the virulence genes (*ace* gene and *gelE* gene) is shown in Figure 5.

### Identification of antimicrobial resistance genes from confirmed *Enterococcus* isolates

The isolates were screened for the eleven (11) genes coding for resistance to antimicrobials in five families (glycopeptides, beta-lactams, tetracycline, flouoroquinnone and macrolides). Among the genes examined for vancomycin resistance, *vanA* and *vanB* were not detected, but *vanC2/3* genes were found. Not all the strains that were phenotypically resistant to vancomycin were positive for *vanA*, *vanB* or *vanC1*. But, Table 5 shows that 6 (9%) and 3 (4.5%) were positive for *ermB* and *vanC2/3* genes, respectively. Thirteen per cent (13%) of the isolates possessed the *gyrA* gene, 1.5% harboured the *ampC* gene, whereas none of the isolates harboured either the *bla*<sub>TEM</sub> or *bla*<sub>Z</sub> gene. Among the tetracycline-resistance genes; *tetK*, *tetL* and *tetM*, the *tetM* gene (n = 20, 30%) was the predominant gene identified, followed by the *tetL* gene. None of the isolates harboured the *tetK* gene. Figure 6 to 10 show images of Gel electrophoresis of detected antimicrobial resistance genes.

### Discussion

The presence of faecal indicator bacteria such as enterococci in recreational water may be indicative of the presence of entero-pathogenic microorganisms such as viruses and some pathogenic bacterial species, including those of the genus *Enterococcus*. In the present study, the presence of *Enterococcus* species was confirmed by PCR techniques targeting the *tuf*- gene. Among the detected virulence genes was the *ace* gene which encodes a collagen adhesion protein active in mediating adherence to host cell epithelia, a virulence strategy mostly associated with infections caused by *E. faecalis* [49]. Antibodies against *ace* have been established on many occasions in serum from patients with endocarditis [50]. Some reports have suggested that genes for virulence factors may be more common in *E. faecalis* than in *E. faecium* [51,52], which confirms the findings of the present study. Among the *E. faecalis* isolates, the most frequently identified virulence gene was *gelE* (15%), followed by *ace* (13. 4%). Wade *et al.* [53] reported a resilient affiliation between enterococci and illness among swimmers in coastal waters. In addition, out of the confirmed *Enterococcus* strains in this study, 96% were resistant to at least three or more of the antimicrobial agents tested, which corroborates the

**Table 2.** Oligonucleotide primers used to identify resistance genes.

Antimicrobial family	Primer	PCR primer sequence (5' -3')	Amplicon size (bp)	PCR conditioncycle	Reference
<b>Glycopeptide</b>	<i>vanA</i>	AF- GCGCGGTCCACTTGTAGATA AR-TGAGCAACCCCAACAGTA	314	94°C, 94°C, 56.5°C, 72° C,72°C 3 min, 1 min, 1 min, 1 min, 10 min 35	Nam <i>et al.</i> , 2012
	<i>vanB</i>	BF-AGACATTCCGGTCGAGGAAC BR-GCTGTCAATTAGTGCGGGAA	220	94°C, 94°C, 56.5°C, 72° C,72°C 3 min, 1 min, 1 min, 1 min, 10 min 35	Nam <i>et al.</i> , 2012
	<i>vanC-1</i>	C1 F-ATCCAAGCTATTGACCCGCT C1 R-TGTGGCAGGATGTTTTTCAT	402	94°C, 94°C, 56.5°C, 72° C,72°C 3 min, 1 min, 1 min, 1 min, 10 min 35	Nam <i>et al.</i> , 2012
	<i>vanC-2/3</i>	C2 F-CTAGCGCAATCGAAGCACTC C2 R-GTAGGAGCACTGCGGAACAA	582	94°C, 94°C, 56.5°C, 72° C,72°C 3 min, 1 min, 1 min, 1 min, 10 min 35	Nam <i>et al.</i> , 2012
<b>Macrolides</b>	<i>erm(B)</i>	BN1CGAGTGAAAAAGTACTCAACA BN2-CGGTGAATATCCAAGGTACG	320	94°C, 94°C, 55°C, 72° C,72°C 3 min, 1 min, 1 min, 1 min, 10 min 35	Okoh, 2009
<b>β-lactam</b>	<i>ampC</i>	F: TTCTATCAAMACTGGCARCC R: CCYTTTTATGTACCCAYGA	550	94°C, 94°C, 60°C, 72° C,72°C 4 min, 45 s, 45 s, 45 s, 7 min 30	Velusamy <i>et al.</i> , 2007
	<i>blaTEM</i>	F: TTTCTGTGTCGCCCTTATTCC R: CCGGCTCCAGATTATCAGC	690	94°C, 94°C, 60°C, 72° C,72°C 5 min, 30 s, 30 s, 90 s, 5 min 30	Jannine <i>et al.</i> , 2010
	<i>blaZ</i>	F: ACT TCA ACA CCT GCT GCT TTC R: TGA CCA CTT TTA TCA GCA ACC	490	94°C, 94°C, 60°C, 72° C,72°C 5 min, 30 s, 30 s, 90 s, 5 min 30	Baddour <i>et al.</i> , 2007
<b>Tetracycline</b>	<i>tetK</i>	F: GTAGCG ACA ATA GGT AAT AGT R: GTAGTG ACA ATA AAC CTC CTA	460	94°C, 94°C, 50°C, 72° C,72°C 5 min, 1 min, 30 s, 30 s,, 5 min 35	Strommenger <i>et al.</i> , 2003
	<i>tetM</i> <i>tetL</i>	F: AGT GGA GCG ATT ACA GAA R: CAT ATG TCC TGG CGT GTC TA F: GTGGTTGCGCGCTATATTCC R:GTGAACGTGACCCACCTAA	158 761	94°C, 94°C, 50°C, 72° C,72°C 5 min, 1 min, 30 s, 30 s,, 5 min 35 94°C, 94°C, 50°C, 72° C,72°C 5 min, 1 min, 30 s, 30 s, 5 min 35	Strommenger <i>et al.</i> , 2003
<b>Flouroquinone</b>	<i>Gyra</i>	F: CGCGTACTATACGCCATGAACGA R: ACCGTTGATCACTTCGGTCAGG	441	95°C, 94°C, 55°C, 72°C, 72°C 3 min, 1 min,1 min, 1.5 min, 5 min35	Yan <i>et al.</i> , 2000

findings of Łuczkiwicz *et al.* [54] who also reported resistant enterococci species from surface water samples. From previous studies, Garido *et al.* [55] highlighted that strains that show resistance to linezolid may also display co-resistance to more antibiotics including chloramphenicol, gentamicin, vancomycin, fluoroquinolones, and macrolides. These authors [55] also reported a high percentage of *E. faecium* and *E. faecalis* isolates that showed resistance to tetracycline which was very similar to our results. In another study conducted by Kimiran-Erdem *et al.* [56], 3% of their

**Table 3.** Patterns of multiple antibiotics-resistance phenotypes (MARPs) of *Enterococcus* species for each sampling location.

Site	Number of antimicrobials	Resistance pattern	Number observed ARI
<b>KP1</b>	5	VA/LZD/CIP/RP/E	1
			0.5
<b>KP2</b>	5	VA/LZD/NIT/RP/E	1
			0.6
	6	VA/LZD/CIP/C/RP/E	1
		VA/CIP/NOR/C/RP/E	1
	7	VA/LZD/CIP/NOR/T/RP/E	1
		AP/VA/LZD/NIT/T/RP/E	1
			1
<b>KP3</b>	3	LEV/NOR/E	1
			0.6
	4	VA/CIP/RP/E	1
		VA/CIP/LEV/RP	1
	6	VA/LZD/NIT/T/RP/E	1
	7	AP/CIP/LEV/NOR/T/RP/E	1
	8	AP/VA/CIP/LEV/NOR/T/RP/E	8
		AP/NIT/CIP/LEV/NOR/T/RP/E	1
		AP/LZD/CIP/LEV/NOR/T/RP/E	1
		VA/LZD/CIP/LEV/NOR/T/RP/E	1
		VA/LZD/NIT/LEV/NOR/T/RP/E	1
			1
			1
<b>KP4</b>	4	VA/CIP/RP/E	2
		VA/LZD/RP/E	0.5
		VA/LZD/NIT/CIP	1
		CIP/LEV/NOR/E	1
	5	NIT/CIP/NOR/RP/E	1
		VA/CIP/NOR/RP/E	1
		VA/LZD/CIP/RP/E	1
	6	VA/LZD/CIP/NOR/RP/E	1
	8	AP/VA/LZD/NIT/T/C/RP/E	1
		AP/VA/NIT/CIP/T/C/RP/E	1
		LZD/NIT/CIP/LEV/T/C/RP/E	1
	9	AP/VA/LZD/NIT/CIP/T/C/RP/E	1
	10	AP/VA/LZD/NIT/CIP/LEV/NOR/T/C/E	1
			1
<b>KP5</b>	4	VA/CIP/RP/E	1
		0.6	

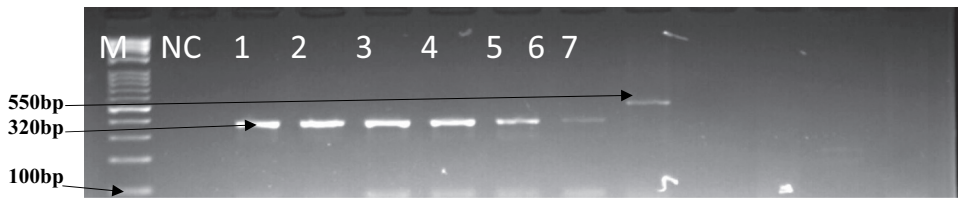
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**Table 3.** (Continued).

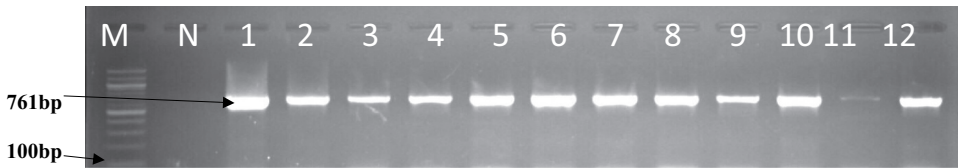
Site	Number of antimicrobials	Resistance pattern	Number observed ARI
	5	VA/LZD/CIP/RP/E	1
		AP/VA/LZD/RP/E	1
	6	CIP/LEV/NOR/T/RP/E	3
		VA/LZD/CIP/T/RP/E	1
		VA/LZD/NIT/CIP/RP/E 1 AP/VA/LZD/NIT/CIP/RP	1
	7	AP/VA/LZD/NIT/C/RP/E	3
		AP/VA/LZD/NIT/T/RP/E	1
		AP/VA/LZD/NIT/CIP/C/RP	1
		AP/CIP/LEV/NOR/T/RP/E	1
	8	VA/LZD/NIT/CIP/NOR/T/RP/E	1
	9	AP/VA/LZD/NIT/LEV/T/C/RP/E	1
		AP/VA/LZD/NIT/CIP/T/C/RP/E	1
	10	AP/VA/LZD/NIT/CIP/LEV/T/C/RP/E	2
<b>KP6</b>	4	VA/LZD/RP/E	1 0.5
	5	VA/LZD/NIT/RP/E	1
	8	AP/VA/RP/NIT/T/C/RP/E	1

seawater isolates showed resistance to chloramphenicol, whereas, chloramphenicol resistance in this study was observed to be 19.9%. It was observed that geographical factors could be responsible for the variances in the resistance.

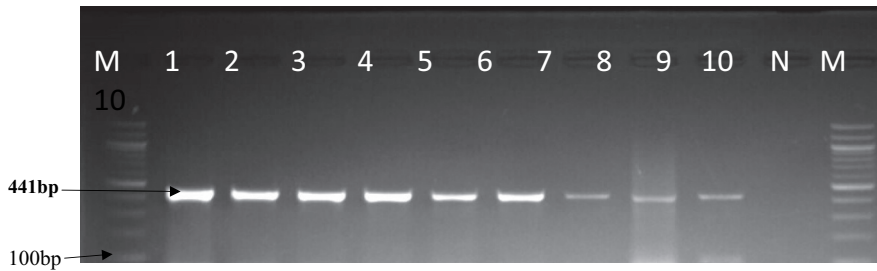
The incidence of antimicrobial-resistant microbes in recreational waters is a major public health problem [57]. Human beings are most likely to be exposed to antibiotic resistance bacteria in coastal water environments that are polluted with faecal wastes, especially during various recreational activities [58,59]. The high level of resistance to erythromycin, rifampicin, tetracycline, linezolid and vancomycin observed could be attributable to the influence of domestic wastes, surface runoff and several human activities in the sea catchment area (Figure 1). The unrestrained use of antibiotics in the treatment of animals and their integration in animal feed has also been presumed to account considerably for the upsurge in antimicrobial resistance in pathogenic bacterial isolates [60,61]. Survival of strains with higher rates of mutation (e.g., bacteria with hypermutator phenotypes) may be a reason why antimicrobials are found in the environment, such as hospitals or intensive farm settings [62–64]. The present study found a high level of resistance to ciprofloxacin (13%). This agrees with the report of Alipour *et al.* [65] who found ciprofloxacin resistance to be 30% among enterococci isolated from river and coastal water. It has been suggested that although enterococci exhibit low levels of intrinsic



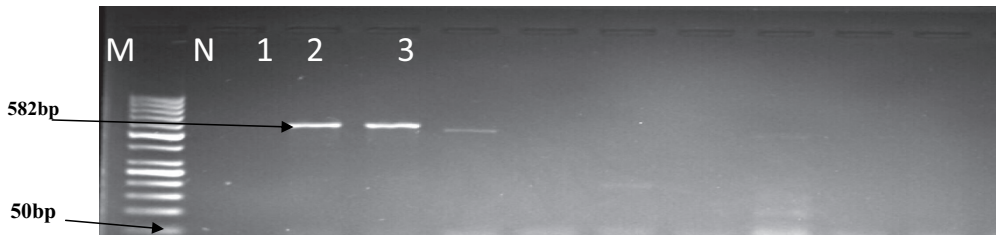
**Figure 7.** A gel electrophoresis showing the resistance determinants of *ermB* and *ampC* gene. Lane M = Molecular ladder (100 bp), NC; negative control, Lane 1–6 positive isolates for *ermB* (320 bp) and Lane 7: *ampC* gene (550 bp)



**Figure 8.** A gel electrophoresis showing the *tetL* gene. M = Molecular ladder (100 bp), N; negative control, lane 1–12 positive isolates. Lane N; negative control, Lane M; Molecular ladder



**Figure 9.** A gel electrophoresis showing the *gyrA* (441 bp) gene. M = Molecular ladder (100 bp), N; negative control, lane 1–10 positive isolates. Lane N; negative control M; Molecular ladder



**Figure 10.** A gel electrophoresis showing the *VanC2/3* (582 bp) gene. M = Molecular ladder (50 bp), N; negative control, lane 1–3 positive isolates.

resistance to quinolones, they can nevertheless develop high-level resistance through various mechanisms such as mutations in genes encoding the target site of fluor-quinolone (DNA gyrase and topoisomerase IV), over-expression of efflux pumps that extrude the drug from the cell, and protection of the FQ target site by a protein designated Qnr family [55,66]. Mutations have also been described in the target genes *gyrA* and *parC* in *E. faecium* and *E. faecalis* [67] leading to continuously increasing levels of resistance [68]. Among some of the most challenging enterococci to handle are the vancomycin-resistant enterococci (VRE) and their detection in the aquatic environments has been documented [37,69,70]. VRE represented 8% (18 of 227) of enterococci isolates from some public beaches in Washington and California [71]. Among the genes examined for vancomycin resistance in this study, *vanA*, *vanB* and *vanC1* were not detected, and *vanC2/3* (n = 3) genes were found in only 4.5% of the isolates. In northern Greece, the *vanC2/C3* type of VRE was similarly recovered from beach waters. This indicates that *vanC* type VRE may be extensively disseminated in aquatic milieus, such as rivers and beach areas [72]. VRE carrying *vanA* and *vanB* are the most essential genotype relating to nosocomial infections [73], though these were not detected in Kidd's Beach water. Several studies have shown that the rigorous use of avoparcin as a growth promoter in animals' nutrition can contribute to a proliferation in VRE [74–76].

The multiple antibiotic resistance (MAR) index shown on Table 4 is a good risk assessment tool and the value of the MAR index of 0.2 has been applied to differentiate low- and high-risk areas where antibiotics are often used, as Riaz *et al.* [77] and Christopher *et al.* [78] have reported. The ARI and MARI in all sampling points on the beach were found to be greater than the 0.2 threshold value, indicating the possible excessive use of antibiotics and greater exposure to antibiotics which may cause high health risk to the water bathers. The ARI in the present study ranged from 0.45 to 0.64. The highest ARI for the tested sampling points were 0.6 at KP3 and 0.64 at KP5, approximately three times the 0.2 limit. This shows that the loads of antibiotics being discharged into these locations are greater than at other points. The increase in the threshold values could be as a result of the socio-economic factors associated with each sampling point. KP1 and KP2 are the major accessible places where swimming, surfing and fishing take place along the beach shore. KP1 and KP2 were also exposed to runoff from the canal during the down-pour, possibly increasing the anthropogenic pollution in the area. KP3 and KP6 are very close to the point where municipal sewage is discharged into the ocean. The various socio-economic activities near the sampling sites suggest that there is a high risk of pollution in using the coastal water for recreation. Incidence of antibiotic-resistant bacteria in a given milieu may be a signal that the area is polluted with antimicrobials [79]. For instance, Al-Bahry *et al.* [80] reported that the major reasons responsible for the marine environment to be contaminated were inappropriate and excessive use of antimicrobial drugs by human beings, including in animal husbandry. Three most important reasons were reported for the emergence of MDR enterococci: baseline intrinsic resistance to numerous antimicrobial agents, acquired resistance, and the transferability of resistance [81].

Generally, the one-way ANOVA showed that ampicillin, vancomycin, linezolid, levofloxacin, rifampicin and norfloxacin were significantly different in their effect against the

isolates from all sites ( $P < 0.05$ ); whereas the resistance profile of the isolates against nitrofurantoin, ciprofloxacin, tetracycline, chloramphenicol and erythromycin were not significantly different ( $P > 0.05$ ). In the present study, all isolates were screened for  $\beta$ -lactams *ampC*, *bla*<sub>TEM</sub> and *bla*<sub>Z</sub> genes. Ampicillin resistance gene *ampC* was detected at a very low rate (Table 5). The low resistance to these  $\beta$ -lactam antibiotics was not astounding, for ampicillin is one of the most frequently used antibiotics for human and animal diseases [82]. The *ampC* gene encoding  $\beta$ -lactams has been identified in the bacteriological strains from wastewater, surface water and even from drinking water films [83].

Tetracycline has led to widely disseminated tetracycline-resistant bacteria [84,85]. Currently, over 20 different *tet* genes have been reported, most of which are harboured in mobile genetic elements [86]. The *tetM* gene is implicated in ribosomal protection while *tetL* codifies an efflux pump; and both of them are commonly reported to be related to tetracycline resistance in enterococci strains [87,88]. In line with previous studies, results of the present research have also shown the presence of *tetL* and *tetM* genes encoding tetracycline resistance and were detected in 19% and 30%, respectively, of the total isolates in the study. Among enterococci species, *tetM* is the most frequently tetracycline-resistance gene encountered. It is most generally situated in the bacterial chromosome and has been found to be linked with conjugative transposons related to the Tn916/Tn1545 family [89,90]. Remarkably, some *Enterococcus* strains exhibiting resistance to tetracycline were negative for *tetM* and *tetL* indicating that these may harbour other *tet* determinants.

In the macrolide class, erythromycin is a common drug and its mechanisms of resistance have been recognised as target enzymes, of which more than 40 *erm* genes have been discovered [91–93]. *ermB* genes happen to be one of the four main classes of *erm* resistance determinants that are correlated with disease-causing microorganism [91]. In this study, 19% of the isolates showed the occurrence of the *ermB* gene signifying that the macrolides resistance in the *Enterococcus* species identified from Kidd's Beach is mainly correlated with the presence of the *ermB* gene. Owing to the clinical importance of ciprofloxacin, Magiorakros *et al.* [94] mentioned it as one of the means considered in MDR assessment tables. In the present study, 13% of the ciprofloxacin-resistant strains showed the *gyrA* gene. In another study, Torell *et al.* [95] reported that 17% of ciprofloxacin-resistant strains contained the *gyrA* gene.

### Conclusion and recommendation

This study presents the first documented evidence for multi-drug resistance and virulence characteristics among *Enterococcus* spp. at Kidd's Beach. The evaluation of *Enterococcus* spp. in the coastal water samples clearly indicates the faecal contamination, which is resistant to various types of antibiotics in coastal waters and may cause illness among swimmers. To track the specific sources of each isolate found at the beach, it would be necessary to map the key determinants of antibiotic resistance in the entire municipality. Moreover, apprehension of the virulence features of circulating *Enterococcus* strains may help to comprehend the complex pathogenic processes of these opportunistic microorganisms. It has not been determined whether these bacteria constitute a threat to bathers through quantitative microbial risk assessment. The existence of antibiotic-resistant enterococci with virulence traits



evaluated in this study suggests that these factors may play a causative role in enterococcal infections. The risk should be subject to regular monitoring to avert the threat of developing systemic disease. The occurrence of antibiotic-resistant *Enterococcus* spp. in coastal waters poses a high risk to public health.

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No potential conflict of interest was reported by the authors.

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