Antibiotic-resistance and virulence genes in *Enterococcus* isolated from tropical recreational waters

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ABSTRACT

The prevalence of enterococci harboring tetracycline- and vancomycin-resistance genes, as well as the enterococcal surface protein (*esp*) has mostly been determined in clinical settings, but their prevalence in tropical recreational waters remains largely unknown. The present study determined the prevalence of *tetM* (tetracycline-resistance), *vanA* and *vanB* (vancomycin-resistance) in the bacterial and viral fractions, enterococci and their induced phages isolated from tropical recreational marine and fresh waters, dry and wet sands. Since lysogenic phages can act as vectors for antibiotic-resistance and virulence factors, the prevalence of the mentioned genes, as well as that of an integrase-encoding gene (*int*) specific for *Enterococcus faecalis* phages was determined. Up to 60 and 54% of the bacterial fractions and enterococci, respectively, harbored at least one of the tested genes suggesting that bacteria in tropical environments may be reservoirs of antibiotic-resistance and virulence genes. *int* was detected in the viral fractions and in one *Enterococcus* isolate after induction. This study presents the opportunity to determine if the presence of bacteria harboring antibiotic-resistance and virulence genes in tropical recreational waters represents a threat to public health.

Key words | antibiotic-resistance, bacteriophages, enterococcal surface protein, enterococci

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INTRODUCTION

There are increasing concerns about bacteria harboring antibiotic-resistance and virulence genes in recreational waters. One of the main reasons is that it remains unknown whether microorganisms harboring these genes are a risk to bathers. Among these, enterococci have received great attention since these are ubiquitous to many aquatic ecosystems (Rivera *et al*. 1988; Devriese *et al*. 1992; Jett *et al*. 1994; Byappanahalli *et al*. 2003; Roberts *et al*. 2009). However, certain *Enterococcus* spp. are also important opportunistic pathogens and infections are usually treated using tetracyclines. Because of this, some studies have focused on tetracycline-resistant enterococci and these have been shown to harbor at least one of the *tet* determinants, *tetM* being one of the most studied in Gram-positive bacteria and the most prevalent in enterococci (Murray 1998; Tannock 1999; Roberts 2005). Resistant *Enterococcus* infections can be treated with vancomycin, but only as a last resort because of the side-effects (Rocha *et al*. 2002). However, enterococci have also acquired resistance to vancomycin due to genes such as *vanA* and *vanB*, commonly described in *E. faecalis* and *E. faecium*. In addition, enterococci exhibiting resistance to antibiotics can harbor genes encoding for virulence factors, as in the case of the enterococcal surface protein (*esp*), described in *E. faecalis* and *E. faecium* (Shankar *et al*. 2001; Toledo-Arana *et al*. 2001; Hammerum & Jensen 2002; Klare *et al*. 2005). The *esp* variant present in *E. faecium* was proposed as a marker of human fecal contamination and there have been conflicting studies on the usefulness of this gene as a species-specific marker (Scott *et al*. 2005; Ahmed *et al*. 2008; Byappanahalli *et al*. 2008; Layton *et al*. 2009).

Another concern is the transmission of antibiotic-resistance and virulence genes by mobile elements, such as plasmids and transposons. However, such studies often ignore the potential of bacteriophages as vectors of such

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genes (Garcia-Vallve *et al*. 2000; Oancea *et al*. 2004). Lysogenic phages can carry additional bacterial genes that could promote fitness and virulence in the recipient bacteria (Hendrix *et al*. 1999, 2000; Hendrix 2003). These genes are often encoded by the host and may be accidentally packaged into the phage capsid. It has been shown that the viral DNA fraction of sewage and environmental waters harbor β-lactamase-encoding genes and that lysogenic phages infecting *E. faecalis* may harbor genes that influence virulence in the bacterial host (Yasmin *et al*. 2010; Colomer-Lluch *et al*. 2011). Most lysogenic phages infecting enterococci described so far belong to the *Siphoviridae* family (dsDNA) and one possible approach to identify these is by amplifying integrase-encoding genes (*int*). Integrases are considered markers of lysogeny and are involved in the integration of the phage genome into that of the bacterial host (Groth & Calos 2004). Detection of phage integrases within an ecosystem may provide insights into what possible risks exist within a specific ecosystem (Heinemann *et al*. 2006).

The prevalence of *tetM*, and *vanA* and *vanB* has mostly been determined in animal husbandry facilities, and temperate and subtropical recreational waters, respectively (Agerso *et al*. 2006; Roberts *et al*. 2009), and to our knowledge, one study has determined the prevalence of *esp* in tropical regions (Betancourt & Fujioka 2009). In addition, the presence of antibiotic-resistance and virulence genes in the bacteriophage fraction of recreational waters and sand, and integrases as a way to identify these, remains largely unknown. The main reason for collecting sand samples is because it can act as a possible reservoir of antibiotic-resistance and virulence genes (Hartz *et al*. 2008; Fernandes Cardoso de Oliveira *et al*. 2010). Therefore, the main aim of the present study was to determine the prevalence of *tetM*, *vanA*, *vanB* and *esp* variants present in *E. faecalis* and *E. faecium*, as well as *int*, in the bacterial and DNA viral fractions, enterococci and their lysogenic phages in tropical recreational environments.

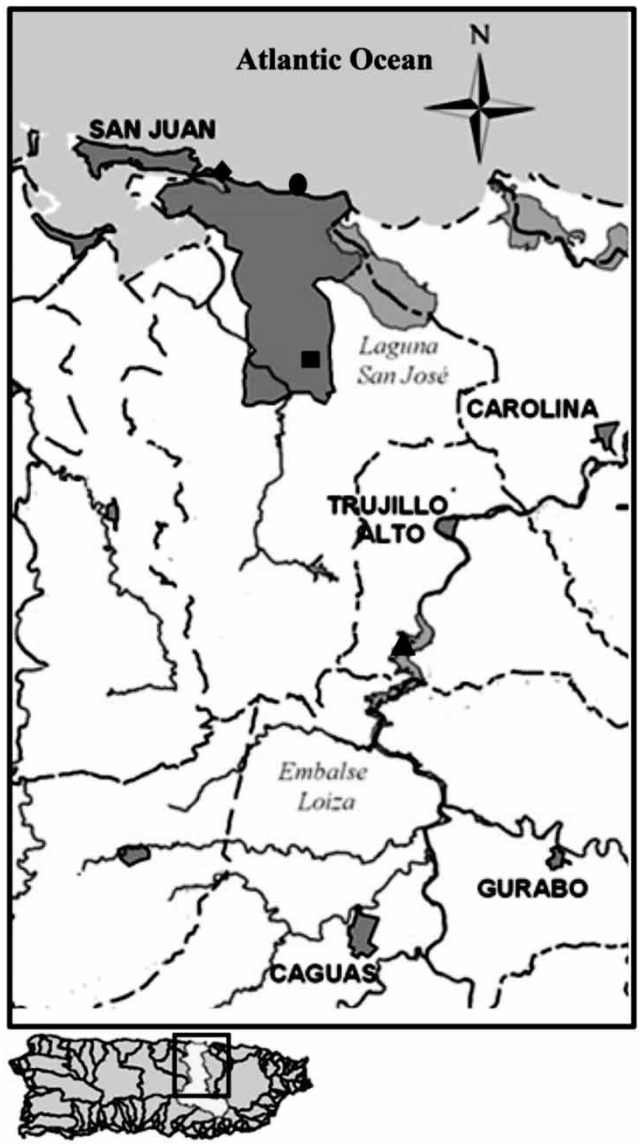

Figure 1 | Sampled sites in this study. Sites included M1 (◆), M2 (•), F1 (■), F2 (▲) and in Puerto Rico. Marine water and dry and wet sands were collected from M1 and M2. Fresh water was collected from F1 and F2.

MATERIALS AND METHODS

Study sites

Samples were collected from several sites in Puerto Rico (Figure 1). For convenience, beaches and fresh water sources in this study will be referred to as marine water 1 (M1) and marine water 2 (M2), and fresh water (F1) and fresh water (F2). M1 and M2 are beaches located in the Atlantic Ocean, alongside the northern area of the island and are heavily used by bathers year-round. Fresh water (F1), which is a man-made lake whose influents are three of the island's major rivers, serves as a major water reservoir for the San Juan metropolitan area and is also used for recreational purposes. Fresh water (F2) receives the input of

minor rivers and streams used for recreational activities. One-liter grab samples of marine (M1, $n = 35$; and M2, $n = 13$) and fresh (F1, $n = 8$; F2, $n = 10$) waters were collected in sterile plastic bottles from July 2011 to May 2012. In addition, 100 g of wet and dry sands from M1 ($n = 16$ and $n = 15$, respectively) and M2 ($n = 14$ and $n = 11$, respectively) were sampled. All water and sand samples were stored at 6–8 °C and processed within 6 h.

DNA extraction of the bacterial fraction and enterococci

To determine the presence of the mentioned genes in the bacterial fraction, 100 mL samples were processed by membrane filtration (0.4 μm pore size, 47 mm diameter) (GE Water and Process Technologies, Trevose, PA, USA). The filter was placed in 25 mL of Azide Dextrose Broth (Difco) and incubated at 37 °C for 24 h. DNA was extracted from 1 mL by using the Fermentas GeneJet Genomic DNA Purification Kit following the manufacturer's instructions. Alternatively, filters were placed in tubes containing 0.3 g of acid-wash glass beads (Sigma) and stored at −20 °C until processed. For this, 600 μL of sodium acetate-EDTA (AE) buffer were added, placed on a bead-beater for 60 s at maximum speed followed by centrifugation for 1 min at 1,000 *g*. The supernatant was collected, re-centrifuged for 5 min at 10,000 *g* to remove any remaining debris and stored at −20 °C until processed (USEPA 2010). For the sand samples, 10 g were directly added into 20 mL of Azide Dextrose Broth, incubated at 37 °C for 24 h and 1 mL was used for DNA extraction using the Fermentas GeneJet Genomic DNA Purification Kit. Alternatively, 10 g were eluted in 120 mL of 0.1% Tween20, 100 mL of the elution were filtered and DNA from the membranes was extracted as described above.

Enumeration of enterococci was performed by using membrane filtration and m-*Enterococcus* agar (Difco) as previously described (USEPA 2002; Santiago-Rodriguez *et al.* 2010). For the detection of enterococci in sand, 10 g were eluted in 0.1% Tween20 followed by filtration of the resulting solution, and membranes were placed on m-*Enterococcus* agar as described. After 24–48 h incubation at 37 °C, individual colonies were picked and transferred onto m-*Enterococcus* plates containing tetracycline (final concentration 16 μg/mL) or vancomycin (final concentration 20 μg/mL). Colonies growing in the presence of these antibiotics were picked and enriched in 1 mL of Azide Dextrose Broth for up to 48 h at 37 °C and DNA was extracted as described above. DNA quality and concentration was estimated by using a NanoDrop® (ND-1000) spectrophotometer.

Virus concentration and DNA extraction

Filtrates from the above were recovered, concentrated and purified for the detection of the mentioned genes in the viral DNA fractions as follows: a final concentration of 0.2% chloroform (v/v) was added to the viral suspensions and kept at room temperature for 30 min to eliminate any remaining viable bacteria. NaCl (enzyme grade >99.0%, Fisher Scientific, NJ, USA) was added to a final concentration of 0.5 M and stored at 4 °C for 1 h. Any remaining bacterial debris was removed by centrifugation at 8,000 *g* for 10 min at 4 °C and the supernatant was transferred to sterile Oakridge tubes. Polyethylene glycol (PEG 8000, Promega, Madison, WI, USA) was added to a final concentration of 10% and stored at 4 °C for 24 h. Precipitates were then sedimented at 10,000 *g* at 4 °C for 15 min and the supernatant discarded. The phage containing pellet was resuspended in 0.5 mL of PBS (pH 7.0) overnight at 4 °C. The residual PEG and bacterial debris were removed by adding an equal volume of chloroform to a Phase Lock Gel tube (5 Prime Inc., MD, USA) and centrifuging at 4,500 *g* for 15 min. Viral DNA was extracted by using phenol:chloroform followed by precipitation with 95% ethanol or the Wizard DNA Clean up system following the manufacturer's instructions (Boulanger 2009). Agarose gels (0.7% w/v) were visualized using GelStar Nucleic Acid Gel Stain (Lonza, Rockland, ME, USA) and were used to confirm molecular weight and purity of the viral DNA.

Lysogen induction

One hundred microliters of overnight culture of 50 randomly selected *Enterococcus* isolates harboring one or more of the genes of interest, were added to 5 mL aliquots of Azide Dextrose (Difco) containing mitomycin C (Sigma) (final concentration of 1 μg/mL). Samples were incubated in a water bath at 37 °C for 4–6 h and centrifuged at 18,000 *g* for 15 min at 4 °C (Raya & Hebert 2009).

Supernatants were tested for the presence of lysogenic phages exhibiting the formation of viral plaques by using the double layer method and *E. faecalis* (ATCC 19433), *E. faecium, E. gallinarum*, *E. hirae* (ATCC 8043), *E. durans* (ATCC 19432), *E. dispar* (ATCC 51266), *E. casseliflavus* (ATCC 25788), *E. pseudoavium* (ATCC 49372) and *Staphylococcus aureus* (ATCC 25923) as the bacterial hosts. Briefly, 100 μL of the possible phage lysates and 1 mL of a fresh culture of each bacterial host were added to 4 mL containing Trypticase Soy Broth (TSB) (Difco) and agar (0.75% w/v) and poured onto a Petri dish containing TSB agar containing $CaCl_2{\cdot}2H_2O$ (Fisher Scientific Co., NJ, USA) and NaN_3 (MCB, OH, USA) (final concentrations of 2.6 and 0.4 mg/mL, respectively). Two temperatures were tested for formation of viral plaques (22 and 37 °C) and enumerated after 24 h. Phages exhibiting the formation of viral plaques were isolated, propagated using *E. faecalis* (ATCC 19433) as the bacterial host and concentrated as described (Bonilla *et al.* 2010). A composite of the phage lysates lacking the formation of viral plaques using the mentioned bacterial strains was concentrated as described. DNA was extracted from phages exhibiting and lacking the formation of viral plaques as described above. One of the induced phage isolates was characterized morphologically by using type-B 200 mesh copper grids placed on top of individual viral plaques and stained with uranyl acetate (UA) 2%, pH 4.5. Bacteriophages were visualized by using a Karl Zeiss Leo 922 energy filtered transmission electron microscope operated at 200 kV.

PCR amplification conditions

All primers used in the present study are described in Table 1. Primer design was performed using Primer 3 (http://frodo.wi.mit.edu/primer3/) and verified for the formation of secondary structures using NetPrimer (http://www.premierbiosoft.com/netprimer/). Detection of *tetM* was performed by designing primers targeting the *tetM* sequence found in GenBank (accession number AY304474.1). For the detection of the *esp* variant of *E. faecalis*, primers were designed using the gene sequence found in GenBank (accession number DQ845099.1). *tetM* and *esp* sequences showed a maximum identity of >98% (*e*-value 0.0) across several bacterial species and isolates. For the detection of lysogenic phages, primers were designed using the integrase gene sequence of the *Enterococcus* phage phiFL1B found in GenBank (accession number GQ478082.1).

Table 1 | Forward (F-) and reverse (R-) primers in this study. Target genes include those encoding for resistance to tetracycline (*tetM*) and vancomycin (*vanA* and *vanB*), the enterococcal surface protein (*esp*) variants found in *E. faecalis* and *E. faecium* and an integrase-encoding gene specific for *E. faecalis* phages (*int*). The 16S rRNA gene amplification was used as a control in the viral fractions and lysogenic *Enterococcus* phages

Target gene	Primer sequence	Amplicon size (bp)	Reference
tetM	F-GGAAAATACGAAGGTGAACA	289	This study
	R-GAATCCCCATTTTCCTAAGT		This study
vanA	F-GGGAAAACGACAATTGC	732	Roberts *et al.* (2009)
	R-GTACAATGCGGCCGTTA		Roberts *et al.* (2009)
vanB	F-TTGCATGGACAAATCACTGC	359	Roberts *et al.* (2009)
	R-GCTCGTTTTCCTGATGGATG		Roberts *et al.* (2009)
esp E. faecalis	F-CACAAATGGGTGAAGGAAGA	490	This study
	R-AGACGAATTTCCCAGTTTGC		This study
esp E. faecium	F-TATGAAAGCAACAGCACAAGTT	680	Scott *et al.* (2005)
	R-ACGTCGAAAGTTCGATTTCC		Scott *et al.* (2005)
int	F-TATTAGGAAAACCTCCGTCA	200	This study
	R-ATATCTTGGGCGTAAGTGAA		This study
16S rRNA	F-AGAGTTTGATCCTGGCTCAG	511	Amann *et al.* (1995)
	R-ACGGGCGGTGTGTRC		Amann *et al.* (1995)

Amplifications were performed in a total volume of 8 μL per reaction, including 1 μL of genomic DNA at a concentration of approximately 10 ng/μL, 4 μL of Fermentas DreamTaq PCR Master Mix, 2.2 μL of MilliQ water and 0.4 μL of each primer at 10 μM. PCR conditions for *tetM* were: an initial denaturation of 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, annealing of 55 °C for 30 s, followed by an extension of 72 °C for 30 s and a final extension of 72 °C for 7 min. Both *vanA* and *vanB* PCR conditions were: an initial denaturation of 96 °C for 3 min, followed by 35 cycles of 96 °C for 30 s, annealing of 60 °C for 1 min, extension of 72 °C for 2 min and a final extension of 72 °C for 10 min. For the detection of the *esp* variant found in *E. faecalis*, the following conditions were used: an initial denaturation of 95 °C for 2 min, followed by 30 cycles of 95 °C for 45 s, annealing of 57 °C for 45 s and an extension of 72 °C for 1 min. Detection of the *esp* variant found in *E. faecium* was done by using an initial denaturation of 95 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, annealing of 58 °C for 1 min and an extension of 72 °C for 1 min. *int* PCR parameters were: an initial denaturation of 95 °C for 2 min, followed by 35 cycles of 94 °C for 45 s, annealing of 55 °C for 45 s, followed by an extension of 72 °C for 1 min and a final extension of 72 °C for 7 min. To ensure that no bacterial DNA was present in the viral DNA, amplification of the 16S rRNA gene was performed using the following PCR conditions: an initial denaturation of 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, annealing of 52 °C for 30 s, followed by an extension of 72 °C for 30 s and a final extension of 72 °C for 10 min. PCR products were visualized in 1.0% agarose gels using GelStar Nucleic Acid gel stain. Positive controls were included for *tetM* and both *esp* variants and potential *int* products were sequenced using an ABI 3130xl Genetic Analyzer.

Statistical analyses

Fisher's exact tests were used to determine differences in the prevalence of the mentioned genes between sample types within the sample sites. The same analysis aimed to determine significant differences in the prevalence of the *esp* variants between sample types. All analyses were performed with the *R* statistical software (v.2.11.1) (Team 2010).

RESULTS

Bacterial fraction and *Enterococcus* isolates

The prevalence of the tested genes in the bacterial fractions of the water and sand samples is shown in Table 2. A significant difference was noted in the prevalence of the *esp* variants of water samples collected from M2 ($p = 0.030$). The prevalence of the *esp* variants and *int* in the *Enterococcus* isolates is shown in Table 3 and that of tetracycline and vancomycin-resistant enterococci and *tetM*, *vanA* and *vanB* is shown in Table 4. Not all enterococci exhibiting resistance to either antibiotic harbored *tetM* or *vanA* and *vanB*. Interestingly, several of these isolates harbored two of the genes of interest. In M1 waters, two of the isolates were positive for both *tetM* and *vanA*, eight harbored *tetM* and the *esp* variant present in *E. faecalis* and one harbored both *vanA*

Table 2 | Prevalence of antibiotic-resistance and virulence-encoding genes in the bacterial fractions of tropical marine (M1, M2) and fresh waters (F1, F2) and wet and dry sands (M1, M2). Percentages are included in parenthesis. Genes included those conferring resistance to tetracycline (*tetM*) and vancomycin (*vanA* and *vanB*), the enterococcal surface protein (*esp*) variants present in *E. faecalis* and *E. faecium* and an integrase-encoding gene specific for *E. faecalis* phages (*int*)

	M1 water ($n = 35$)	M1 wet sand ($n = 16$)	M1 dry sand ($n = 15$)	M2 water ($n = 13$)	M2 wet sand ($n = 14$)	M2 dry sand ($n = 11$)	F1 ($n = 8$)	F2 ($n = 10$)
tetM	6 (17)	2 (13)	2 (13)	4 (31)	1 (7)	1 (9)	2 (25)	2 (20)
vanA	7 (20)	4 (25)	2 (13)	ND[a]	1 (7)	2 (18)	2 (25)	ND[a]
vanB	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
esp E. faecalis	2 (5)	2 (13)	2 (13)	7 (54)	7 (50)	4 (36)	2 (25)	6 (60)
esp E. faecium	6 (17)	4 (25)	5 (33)	1 (8)	ND[a]	ND[a]	ND[a]	ND[a]
int	4 (11)	1 (6)	1 (7)	2 (15)	1 (7)	ND[a]	3 (38)	2 (20)

[a]ND = not detected.

Table 3 | Prevalence of the *esp* variants present in *E. faecalis* and *E. faecium* and *int* in tropical marine (M1and M2) and fresh waters (F1 and F2) and wet and dry beach sands in the enterococci isolates. Percentages are presented in parenthesis

	M1 water ($n = 99$)	M1 wet sand ($n = 55$)	M1 dry sand ($n = 46$)	M2 water ($n = 25$)	M2 wet sand ($n = 21$)	M2 dry sand ($n = 25$)	F1 ($n = 19$)	F2 ($n = 66$)
esp E. faecalis	10 (10)	1 (2)	1 (2)	2 (8)	1 (5)	ND[a]	ND[a]	6 (9)
esp E. faecium	2 (2)	6 (11)	3 (7)	2 (8)	2 (10)	5 (20)	ND[a]	5 (8)
int	3 (3)	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	3 (16)	ND[a]

[a]ND = not detected.

Table 4 | *Enterococcus* isolates tested for tetracycline (16 μg/mL) and vancomycin (20 μg/mL) resistance isolated from tropical samples. The presence of tetracycline (*tetM*) and vancomycin (*vanA* and *vanB*) resistance genes was also tested in the isolates. Percentages are shown in parenthesis

Sample	Antibiotic tested	Tetracycline	Vancomycin	Gene tested	*tetM*	*vanA*	*vanB*
M1 water	188	26 (14)	18 (10)	99	13 (13)	2	ND[a]
M1 wet sand	75	14 (19)	10 (13)	55	9 (16)	ND[a]	ND[a]
M1 dry sand	136	31 (23)	31 (23)	46	9 (20)	ND[a]	ND[a]
M2 water	65	5 (8)	3 (5)	25	2 (8)	ND[a]	ND[a]
M2 wet sand	75	23 (31)	8 (11)	21	2 (10)	ND[a]	ND[a]
M2 dry sand	130	74 (57)	16 (12)	25	13 (52)	ND[a]	ND[a]
F1	173	50 (29)	70 (40)	19	3 (16)	ND[a]	ND[a]
F2	87	22 (29)	24 (28)	66	3 (5)	ND[a]	ND[a]

[a]ND = not detected.

and the *esp* variant found in *E. faecalis*. In wet sands collected from M1 and M2, two and one of the isolates were positive for *tetM* and the *esp* variant present in *E. faecium*, respectively. In dry sands collected from M2, three of the enterococci isolated were positive for *tetM* and the *esp* variant present in *E. faecium*. Similarly, one of the isolates from F1 was positive for the latter combination of genes (data not shown). Significant differences were noted for the prevalence of *tetM* in M2 waters and dry sand ($p = 0.0015$) and M2 wet and dry sands ($p = 0.0038$). The prevalence of both *esp* variants in waters collected from M1 was significantly different as well ($p = 0.033$).

Viral fraction and induced phages

None of the antibiotic-resistance or virulence genes was detected in the viral DNA fractions. None of the samples was positive for the 16S rRNA gene and only *int* was detected in one of the filtrates. Similarly, the antibiotic-resistance and virulence genes tested were not detected in the induced enterococci phages. Not all induced enterococci phages were able to infect most of the *Enterococcus* or *S. aureus* type strains in this study, but those that formed viral plaques were able to replicate at 22 and 37 °C in *E. faecalis*. In addition, enterococci phages induced from *Enterococcus* isolates exhibited two different viral plaque morphologies (data not shown). Some plaques were turbid on the edges and translucent in the center, while other plaques were completely translucent, and these phages were not positive for the integrase-encoding gene tested in this study. On the other hand, one of the induced enterococci phages that did not form viral plaques using the mentioned enterococci type strains was positive for *int*. Sequencing of *int* showed a 99% similarity (*e* value $= 4 \times 10^{-81}$) with *int* of *E. faecalis* phages phiFL1A, 1B, 1C, 2A, 2B, 3A and 3B when performing a BLAST search. In terms of the morphology of one of the induced enterococci phages, this exhibited an icosahedral capsid and a flexible tail of approximately 80 and 180 nm, respectively (Figure 2).

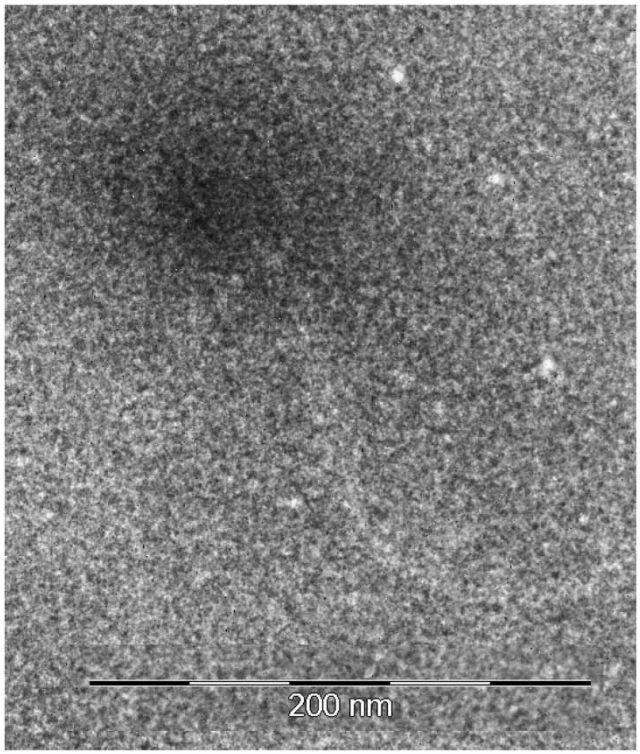

Figure 2 | Characterization of an induced enterococci phage. Induced phage showed a flexible tail of approximately 180 nm and an icosahedral capsid of 80 nm (bar = 200 nm).

DISCUSSION

Many studies focusing on the prevalence of *tet* determinants in the environment have been done in waters associated with swine production facilities and livestock (Aminov *et al.* 2001; Chee-Sanford *et al.* 2001; Agerso *et al.* 2006). However, few studies that focus on the prevalence of tetracycline-resistance genes in tropical recreational waters are available. In the present study, *tetM* was detected in both the bacterial fractions and enterococci isolated from waters and wet and dry sands. Interestingly, significant differences were noted in the prevalence of *tetM* in waters and wet and dry sands collected from M2. This may suggest that the prevalence of *tetM* may be independent of the sample type, but this was not the case for all the sampled sites. The detection of tetracycline-resistant bacteria at the sites tested may suggest that these are being introduced via human fecal material (as there are no animal husbandry facilities in proximity). Another possibility is that the constant presence of tetracycline in waters is selecting for resistant bacteria. This is reasonable since it has been shown that tetracycline can be introduced via feces and urine and detected at concentrations of approximately 0.10 μg/L (Lindsey *et al.* 2001; Zhu *et al.* 2001). The horizontal transfer of genes conferring tetracycline-resistance via mobile elements between autochthonous and allochthonous bacteria is likely, but in tropical recreational waters it may be difficult to identify enterococci from a fecal source and those from the environment. Interestingly, some *Enterococcus* isolates exhibiting resistance to tetracycline were negative for *tetM* suggesting that these may harbor other *tet* determinants.

This is also one of the few reports on the detection of *vanA* and *vanB* in tropical recreational waters. Results suggest that several bacterial species in tropical environments may harbor *vanA* or *vanA*-like genes (e.g. *S. aureus*) (Zhu *et al.* 2008). The present study is comparable with previous results in which enterococci harboring *vanA* have been isolated from marine waters (Novais *et al.* 2005; Roberts *et al.* 2009). In contrast, *vanB* was not detected in any of the samples tested, but this is similar to results presented elsewhere (Schwartz *et al.* 2003). Although the reasons for this remain to be determined, it is possible that harboring *vanA*, instead of *vanB*, may be more beneficial since harboring *vanA* confers resistance to vancomycin and teicoplanin (Cetinkaya *et al.* 2000). It should be noted that most of the *Enterococcus* isolates that exhibited resistance to vancomycin lacked *vanA* or *vanB*. Thus, similarly to *tet*, some of the *Enterococcus* isolates may harbor other genes conferring resistance to vancomycin (e.g. *vanC*, *vanD* and *vanE*); although it is also feasible that genes conferring resistance to other antibiotics may also be conferring resistance to vancomycin. Similar outcomes have been reported in Europe in which vancomycin-resistant enterococci have been associated with the use of avoparcin (Bager *et al.* 1997). The source of *vanA* in tropical waters and sand remains to be determined, although the input of fecal matter remains a possibility since vancomycin-resistant bacteria have been detected in sewage effluents (Novais *et al.* 2005). As with tetracycline, future studies need to determine if vancomycin is being introduced into the waters tested and if it is a selective force in tropical environments.

Detection of both *esp* variants in the bacterial fractions and *Enterococcus* isolates of several of the samples may suggest an input of human and animal fecal material

(Hammerum & Jensen 2002). The absence of the *E. faecium* variant in the bacterial fractions and in enterococci of several of the samples evaluated was not expected since these sites are visited throughout the year and may not be free from fecal contamination from non-point sources. This may suggest that the *E. faecium* variant may not necessarily indicate human fecal pollution in all water types, especially in the tropics, in which enterococci are naturally found in many water bodies (Muñiz *et al.* 1989). Only the bacterial fraction of waters collected from M2 and the enterococci isolated from M2 water exhibited a difference in the prevalence of both *esp* variants. This may suggest that, in tropical environments, similar results are obtained when detecting either *esp* variant.

In the present study, none of the tested genes was detected in the viral DNA fraction or the induced enterococci phages. However, the presence of other antibiotic-resistance and virulence-encoding genes in bacteriophages should not be ignored. In terms of *int*, although it was detected in the *Enterococcus* isolates and one of the induced phages, it is possible that other *Enterococcus* phages may harbor *int* sequences different from the variant tested. Phages infecting enterococci have exhibited differences in their lysogeny modules and *int* sequences (Yasmin *et al.* 2010; Stevens *et al.* 2011). Future studies would need to design primers targeting specific integrase genes, as integrase do not possess extensive conserved nucleotide sequences among different phage families. This study also suggests that *Enterococcus* isolates may harbor more than one inducible prophage, but the function of various prophages in the genomes of enterococci remains to be determined (Banks *et al.* 2003; Brussow *et al.* 2004).

CONCLUSIONS

This is one of the first reports which aimed to identify a battery of antibiotic-resistance and virulence genes in both the bacterial and viral fractions of tropical recreational waters and sand. The presence of antibiotic-resistance and virulence genes in the environmental microbiota of tropical samples suggests that the transmission of these genes is not restricted to strains of the clinical setting. The exact mechanisms of horizontal transfer of antibiotic-resistance and virulence genes under these environmental conditions need to be determined, as do the role of environmental microbiota as reservoirs of these genes. By detecting *int* in lysogenic phages, further studies could determine the presence of antibiotic-resistance and virulence factors in their genomes. Our data also point to the need to take into consideration lysogenic phages when determining the presence or absence of bacterial viruses in the environment.

Most epidemiological studies on recreational waters have focused on gastrointestinal illness; although a few recent ones have also included eye, skin and ear infections. The present study showed a relatively high prevalence of *Enterococcus* spp. exhibiting resistance to antibiotics which also harbored virulence genes. It remains to be determined whether these bacteria represent a risk to bathers and if these are specifically involved in skin infections. However, it is worrying that opportunistic pathogens involved in skin infections and harboring antibiotic resistance are present in recreational waters and sands, where bathers may be exposed to them.

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