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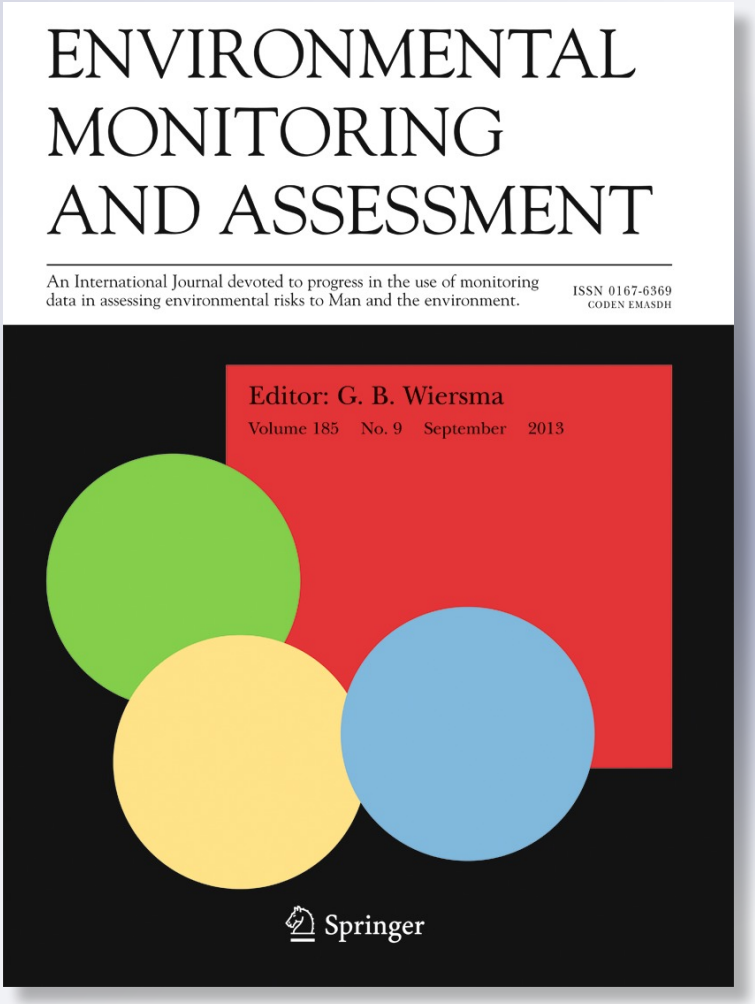
Environmental Monitoring and Assessment
An International Journal Devoted to Progress in the Use of Monitoring Data in Assessing Environmental Risks to Man and the Environment

ISSN 0167-6369
Volume 185
Number 9

Environ Monit Assess (2013)
185:7427-7443
DOI 10.1007/s10661-013-3110-x



Springer

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High-level aminoglycoside resistance and virulence characteristics among *Enterococci* isolated from recreational beaches in Malaysia

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Received: 20 July 2012 / Accepted: 18 January 2013 / Published online: 16 February 2013

Abstract We report the first study on the occurrence of high-level aminoglycoside-resistant (HLAR) *Enterococci* in coastal bathing waters and beach sand in Malaysia. None of the encountered isolates were resistant to high levels of gentamicin (500 μg/mL). However, high-level resistance to kanamycin (2,000 μg/mL) was observed in 14.2 % of tested isolates, the highest proportions observed being among beach sand isolates. High-level resistance to kanamycin was higher among *Enterococcus faecalis* and *Enterococcus faecium* than *Enterococcus* spp. Chi-square analysis showed no significant association between responses to tested antibiotics and the species allocation or source of isolation of all tested *Enterococci*. The species classification of encountered *Enterococci* resistance to vancomycin was highest among *Enterococcus* spp. (5.89 %) followed by *E. faecium* (4.785) and least among *E. faecalis*. A total of 160 isolates were also tested for virulence characteristics. On the whole, caseinase production was profoundly highest (15.01 %) while the least prevalent virulence characteristic observed among tested beach *Enterococci* was haemolysis of rabbit blood (3.65 %). A strong association was observed between the source of isolation and responses for each of caseinase (C=0.47, V=0.53) and slime (C=0.50, V=0.58) assays. Analysis of obtained spearman's coefficient showed a strong correlation between caseinase and each of the slime production (p=0.04), gelatinase (p=0.0035) and haemolytic activity on horse blood (p=0.004), respectively. Suggestively, these are the main virulent characteristics of the studied beach *Enterococci.* Our findings suggest that recreational beaches may contribute to the dissemination of *Enterococci with* HLAR and virulence characteristics.

Keywords Enterococcus · High-level aminoglycoside resistance · Recreational beach · Beach sand · Malaysia

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Introduction

Beach water and sand quality monitoring has attracted significant attention in recent years owing to improved legislation (Casas et al. 2011). Understandably developed nations have institutional frameworks, scientific, managerial and engineering competencies, considerable

budgetary allocations and relevant political and stakeholder willpower to ensure that robust surveillance systems are put in place (Ongley 1997). Yet in these countries, there have been accounts reported of alarming levels of bacteria found in faeces and dangerous heavy metals in contaminated storm water flowing into beaches (Oshiro and Fujioka 1995; Olapade et al. 2006; Grant et al. 2001; Novais et al. 2005; Yoder et al. 2004; Guzmán and Jiménez 1992; Debacker et al. 2000). This often results in a number of public health advisories and beach closures (Graham et al. 2009; Casas et al. 2011).

In less economically developed countries where these institutions are frail or do not exist and budgets are tight (Ongley 1997), prevailing absence of surveillance schemes may allow undetected pollution from storm water, domestic sewage and industrial effluents (Kuylenstierna et al. 2009). Recent review articles on recreational beaches in Malaysia have highlighted potential impacts of tourism activities, shipping, refinery effluent, land reclamation, coastal zone property development and insufficient sewage water treatment as the core factors affecting recreational water quality (Dada et al. 2012b; Praveena et al. 2011). Major efforts have been put in place by relevant agencies in Malaysia to ensure yearly monitoring and public disclosure of pollutants in a number of rivers in Malaysia. Furthermore, a surveillance program is currently in place for marine water quality (DOE 2006) that lays emphasis on levels of *Escherichia coli*, oil and grease, total suspended solids and selected heavy metals.

Public recreational beaches are, however, apparently left out of these surveillance schemes. A recent report highlighted challenges in the management of coastal recreational beaches in Malaysia (Dada et al. 2012b). Particularly worrisome is the possibility of direct sewage and storm water discharge (Fig. 1) coupled with the apparent absence of early warning systems that inform the public on how safe the beaches are. For example, there are no awareness programs that emphasize the need to avoid swimming in the vicinity of storm water drains or to entirely avoid swimming during storms and a few days after. In a previous report, a storm water drainage pipe at Port Dickson was implicated in the preponderance of antibiotic resistant organisms in the recreational beach (Dada et al. 2013a).

In order to ensure that the public is protected from illnesses arising from direct discharge of pollutants from the environment, one important indicator used in the surveillance of bacteriological quality aspects of marine recreational beaches is *Enterococci*. High *Enterococci* levels indicate that there may be more serious pathogens in the water, which could cause respiratory illnesses, diarrhea and skin irritations (Wade et al. 2003). In countries where uncontrolled discharge of domestic and industrial waste is unchecked, the environment may present itself as a reservoir for the selection of antibiotic-resistant and potentially virulent bacteria (McLellan et al. 2007). This is primarily because these wastes are usually rich in antibiotic-resistant bacteria, which may elicit varying levels of virulence depending on the vulnerability of their hosts (Dang et al. 2008). As antibiotic resistance and virulence are major concerns in clinical practice determining outcomes of pathogen exposure and infection (Cosgrove 2006); studies that focus on these characteristics particularly among *Enterococci* are necessary.

Enterococci easily acquire and transfer resistance genes (Kwon et al. 2012) thus increasing the chances of the emergence of *Enterococci* with high-level aminoglycoside resistance (HLAR) (Fernandez et al. 2011). It has been reported that the synergistic effects obtained by the combination of aminoglycosides with penicillin or vancomycin disappear in strains that show high-level aminoglycoside resistance (Fraimow and Tsigrelis 2011; Mendiratta et al. 2008). HLAR can be transmitted within hospitals, through the hands of health care workers (Arias and Karchmer 2011; Hatcher et al. 2012). However, considering the vulnerability of seawater to waste discharge and the reports of HLAR genes in water environment (Schwartz et al. 2003; Tennstedt et al. 2005; Zhang et al. 2009), this study was undertaken to determine the occurrence of HLAR *Enterococci* isolates in recreational waters in Malaysia. To the best of our knowledge, this is the first study that focuses on the occurrence of high-level aminoglycoside-resistant *Enterococci* in recreational bathing waters in Malaysia. In the present study, we also attempted to elucidate phenotypic virulence characteristics among *Enterococci* recovered from the considered beaches.

Materials and methods

Study area

The two beaches considered for sampling during this study are locations selected as part of a nationwide

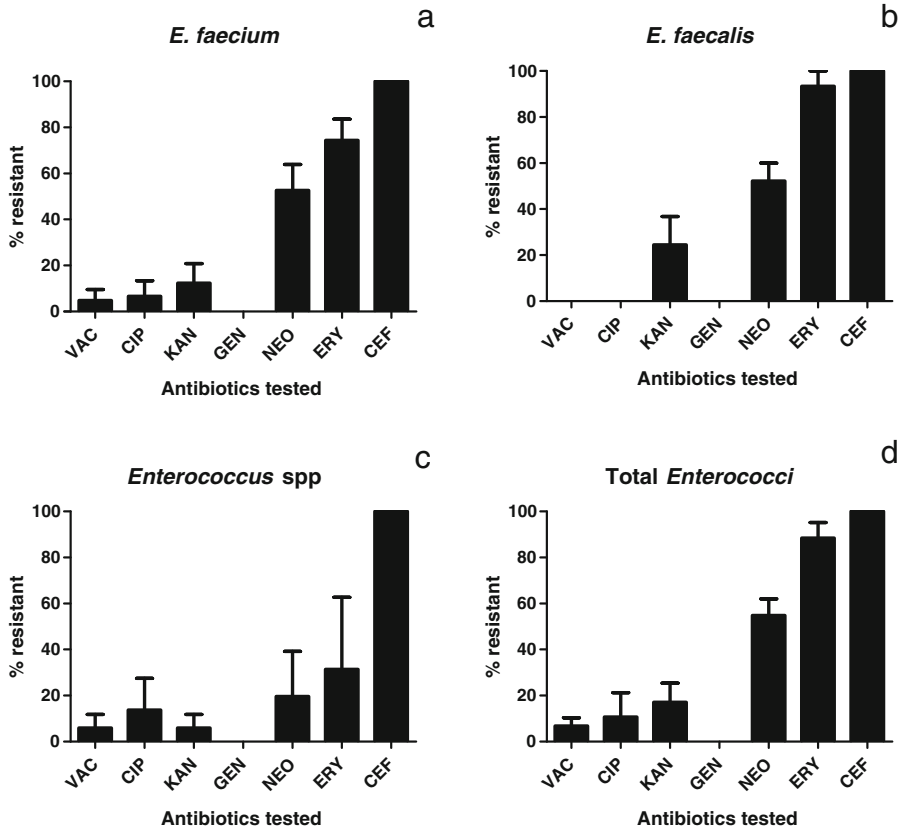

Fig. 1 **a**–**d** Antibiotic resistance based on source of isolation of tested *Enterococci*. Vancomycin (*VAC*), ciprofloxacin (*CIP*), kanamycin (*KAN*), gentamycin (*GEN*), neomycin (*NEO*), erythromycin (*ERY*), cefuroxime (*CEF*)

pathogen monitoring program under the auspices of the School of Bioscience and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. Port Dickson beach is a holiday destination with an estimated population of visitors of up to 20,000 and 50,000 people (Anon 2011). A few studies have documented that there are several sewage discharge pipes that drain directly into this beach (Hamzah et al. 2011). On the other hand, no study has reported sewage pipe discharges at the second sampling location, Bagan Lalang, a coastal beach in the Sepang district in the state of Selangor.

Sample collection

Sterile glass bottles (1,000 mL) were used to collect water samples in duplicates, while sterile disposable containers (500 mL) were used to collect sediments. No sediments were collected from Port Dickson beach. Samples were stored on ice until analysed, usually within 4 h after collection. These included Teluk Kemag beach water sample (PD), Bagan Lalang beach water sample (WS4), Bagan Lalang overland flow water sample (WS2), swamp water sample (WS1), dry beach sand sample (SS4) and wet beach sand sample (WTSD) at Bagan Lalang. Details of the sampling locations are as previously described (Dada et al. 2013b).

Isolation and Identification of *Enterococci*

Enterococci were recovered from seawater using the membrane filtration method as described by APHA (1999) using Slanetz and Bartley (S + B) culture media. A total of 165 colonies of presumptive *Enterococci* were randomly chosen. Using previously published guides (Facklam and Collins 1989; Facklam and Elliott 1995; Facklam 2002) as a guideline, biochemical tests were conducted on the selected isolates. Isolates were tested to confirm if they hydrolyse bile esculin and grow in 6.5 % NaCl and in brain heart infusion agar (BHIA) at 45 °C. Other tests were to determine motility on SIM agar (Oxoid, UK) and fermentation of a 1 % concentration of mannitol, sorbitol, arabinose, raffinose, sucrose, lactose and inulin. Isolates were also confirmed using API 20Strep kits

(bioMerieux, USA). Identities of selected isolates were confirmed by PCR. The 16S rRNA primers used, B27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and U1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) were as previously published (James 2010).

High-level aminoglycoside resistance screening of *Enterococci*

Fifty-five isolates recovered from Port Dickson and Bagan Lalang beach sand samples were selected for antibiotic susceptibility test by agar dilution method using three aminoglycosides: gentamicin, neomycin and kanamycin. Four non-aminoglycoside antibiotics were also included in the study. Concentrations used are gentamicin (4–1,000 μg/mL), neomycin (4–1,000 μg/mL), erythromycin (4–500 μg/mL), kanamycin (1,000 μg/mL), ciprofloxacin (0.6–5 μg/mL), vancomycin (4–125 μg/mL) and ceftriaxone (4–1,000 μg/mL). Screening was as described by Sahm et al. (1991). Individual isolates were spot inoculated at a concentration of approximately 10^6 cfu in 10 μL aliquot of culture on BHIA plates supplemented with known concentration of the tested antibiotics. Unsupplemented BHIA were regarded as the control. Cultures were examined after 24 and 48 h of incubation at 37 °C (Louie et al. 1992). As described by Sahm et al. (1991), any form of weak growth or one or more colonies were interpreted as resistant. *Enterococci* isolate with an MIC=2,000 μg/mL for kanamycin and ≥500 μg/mL for gentamicin was considered as high-level resistant to these aminoglycosides. For other antibiotics, isolates were considered resistant based on the breakpoint interpretive criteria published by the Clinical and Laboratory Standard Institute (CLSI 2008) guidelines. Identities of high-level aminoglycoside isolates were reconfirmed after the HLAR screening.

Virulence assays

One hundred and sixty-five isolates were tested for phenotypic virulence characteristics. These included:

Assay of gelatinase activity Gelatinase assay was as described by Cariolato et al. (2008). In this assay, brain heart infusion agar (BHIA) supplemented with 10 g/L peptone and 30 g/L gelatin was used as the growth medium. After streaking, plates were incubated overnight at 37 °C and then placed at 4 °C for 5 h for subsequent examination of diffuse zone of turbidity around the colonies indicating hydrolysis of gelatine.

Assay of haemolytic activity The haemolytic activity of *Enterococci* isolates was assessed using the method described by Brenden and Janda (1987). In a previous study, it was observed that *Enterococci* isolated failed to lyse sheep blood. Horse and rabbit blood was thus used in this study. Blood agar plates were prepared with Mueller–Hinton agar (MHA, BioLife, Italy) containing defibrinated horse and rabbit blood (final blood concentration 5 %*v*/*v*). After plates were streaked by the tested isolates, an observation of the clear haemolysis zone around colonies after incubation for 24 h at 37 °C indicated haemolytic activity of the isolate.

Assay for caseinase production Casein hydrolysis was assayed using the method described by Archimbaud et al. (2002). For each isolate tested, a 10-μL suspension was streaked on MHA containing 3 % (*w*/*v*) skimmed milk after which the plates were incubated at 37 °C for 24 h. Caseinase activity was observed as the presence of a transparent zone around the colonies.

Assay for qualitative biofilm formation Qualitative biofilm formation was enumerated using a form of phenotypic characterization that elucidates bacteria capable of producing slime as described by Kouidhi et al. (2011) and Arciola et al. (2002). BHIA supplemented with 3.6 % (*w*/*v*) saccharose (Sigma) and 0.08 % (*w*/*v*) congo red dye was used in the assay. Tested isolates were streaked on these plates and incubated at 37 °C for 24 h under aerobic conditions. Isolates whose streak produced any shade of black colonies on the agar were considered to be normal slime-producing strains while red colonies were taken as non-slime-producing *Enterococci*.

Statistical analysis As the response of virulence tests (positive or negative) was mutually exclusive, two-way chi-square analysis was done to suggest evidence of relationships between response to virulence assay and source of isolation. The species distribution of *Enterococci* and its association with response obtained in the virulence assays and high-level aminoglycoside resistance screening was also evaluated using chi-

square test (GraphPad Prism Software, San Diego, CA). One-way ANOVA using Tukey–Krumer comparison tests was used to evaluate differences in proportions of isolates positive to each virulence assay for all species recovered from the water samples (GraphPad Prism Software, San Diego, CA). Differences were considered statistically significant when p values were less than 0.05. Plots were generated using GraphPad software.

Results

A total of 160 isolates were recovered from the tested water samples. Results of speciation of recovered isolates have been published in our previous report (Dada et al. 2013b). In the present study, all species other than *Enterococcus faecalis* and *Enterococcus faecium* were designated as *Enterococcus* spp. Out of these, 55 isolates were selected for high-level aminoglycoside resistance screening. High-level resistance to kanamycin was notably higher among *E. faecalis* and *E. faecium* than *Enterococcus* spp. (Fig. 1a–d). Chi-square analysis showed no significant association between responses to tested antibiotics and the species classification or source of isolation of all tested *Enterococci*. The species classification of encountered *Enterococci* resistance to vancomycin was highest among *Enterococcus* spp. (5.89 %) followed by *E. faecium* (4.78 %) and least among *E. faecalis*. On the whole, one-way analysis of variance revealed significant differences in the proportion of isolates resistant to each antibiotic as compared to the rest. The proportion of resistant *Enterococci* obtained for each of the tested antibiotics is presented in Fig. 2a–d. While a generally higher proportion of resistant bacteria was observed among PD isolates, a higher erythromycin resistance was observed among BL isolates. For all three locations considered, none of the encountered isolates were resistant to high levels of gentamicin (500 μg/mL). While high-level resistance to kanamycin (2,000 μg/mL) was observed in 14.2 % of the tested isolates, the highest proportions observed were among beach sand isolates. Although no vancomycin-resistant isolates were recovered from Bagan Lalang beach water, a low level of vancomycin resistance (5.88 %) was observed among Port Dickson isolates (Fig. 2). All isolates regardless of the source of isolation were resistant to ceftriaxone. It was observed that the drug was ineffective even at high concentrations of up to 500 μg/mL.

On an inter-species basis, the percentage of strains demonstrating virulence characteristics in the current study was highest among observed encountered *Enterococci* spp. (15.57 %). As a whole, among all the tested *Enterococci*, caseinase production was profoundly highest with up to 40 % being positive while the least prevalent virulence characteristics observed was haemolysis of rabbit blood (12.63 %) (Table 1).

An attempt was also made to test for association between species diversity and virulence characteristics observed in the study. While there was significant association between the class of species tested and response observed to gelatinase (χ^2=22.30, df=2, P value<0.0001) and horse blood haemolysis (χ^2= 7.333, df=2, P value=0.00256) assays, no significant association was observed for the other tested virulence characteristics. The strength of the observed relationship for gelatinase and haemolysis assays and the class of species tested was further obtained using Cramer's C and V statistics (Crewson 2006; Twikirize and O'Brien 2012) given by Eq. (1) and (2) below:

$$C = \sqrt{\frac{\chi^2}{n_T + \chi^2}} \tag{1}$$

$$V = \sqrt{\frac{\chi^2}{n(q-1)}} \tag{2}$$

where n_T = total number of isolates tested for the particular virulence assay, n is the number of isolates and χ^2 is the value of chi-square obtained for the analysis. From the results obtained, the amount of variance is explained in the relationship between the response of the virulence assay tested and the source of isolation or the specie allocation of the tested isolate. In which case, the strength of relationship is given by possible responses varying from 0 (poor association) to >0.5 (strong relationship). From these substantiation tests, while a poor association (C=0.2, V=0.21) existed between specie allocation and response to Haem-H assay, a higher but moderate association (C=0.35, V=0.37) was observed between specie allocation and response to gelatinase assay.

Comparisons of means of positive isolates for each virulence assay among the five sampling locations are

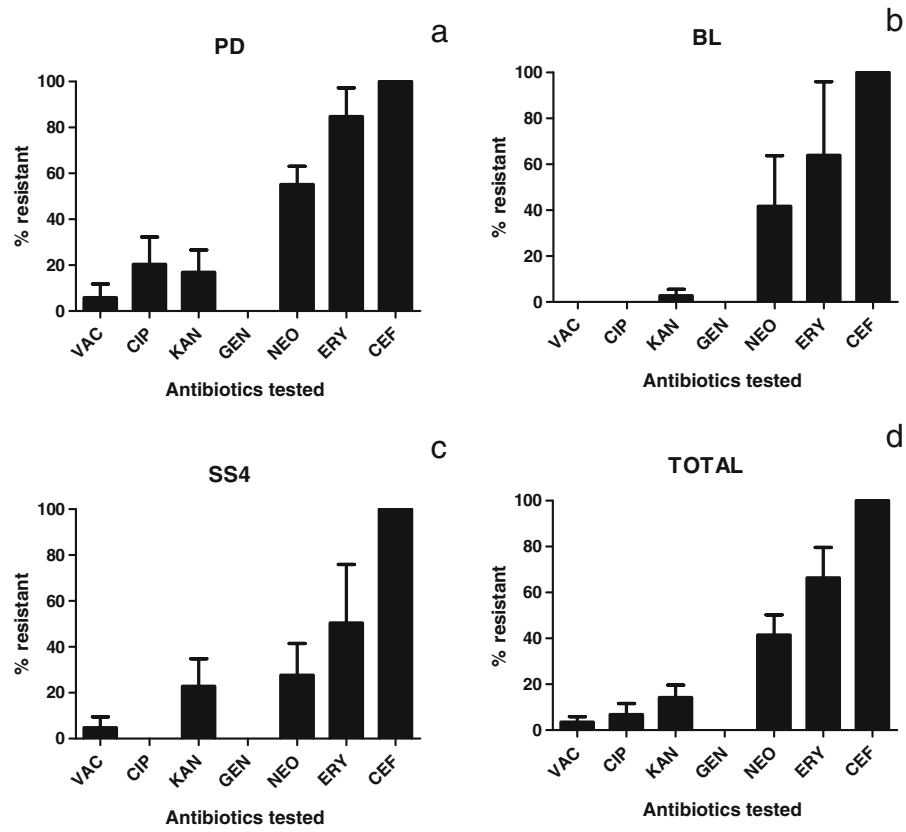

Fig. 2 **a–d** Antibiotic resistance based on source of isolation of tested *Enterococci*. Vancomycin (*VAC*), ciprofloxacin (*CIP*), kanamycin (*KAN*), gentamycin (*GEN*), neomycin (*NEO*), erythromycin (*ERY*), cefuroxime (*CEF*), water sample collected from the bathing area of the beach at Bagan Lalang (BL), dry soil sample collected from the beach sand at Bagan Lalang (*SS4*), water sample collected from bathing area at Teluk Kemang beach, Port Dickson (*PD*)

presented in Fig. 3a–e. Virulence characteristics were consistently highest among isolates recovered from Teluk Kemang beach and lowest among isolates recovered from wet sand (Fig. 3). Chi-square tests revealed association between the source of isolates and response to virulence assay for caseinase (χ^2=44.33, df=5, *P* value<0.0001), slime production(χ^2=54.29, df=5, *P* value<0.0001), gelatinase (χ^2=16.24, df=5, *P* value=0.0062) and Haem-H (χ^2=23.66, df=5, *P* value=0.0003) assays. Substantiation analysis of this association using Cramer's Statistics revealed that response to Haem-H (*C*=0.36, *V*=0.38) and gelatinase (*C*=0.30, *V*=0.32) assays was only mildly associated with source of isolates. However, a stronger association was observed between the source of isolation and responses for each of caseinase (*C*=0.47, *V*=0.53) and

Table 1 Virulence characteristics and species allocation of tested beach *Enterococci*

Virulence characteristics	Species allocation of tested *Enterococci*			
	E. faecalis	*E. faecium*	*Enterococcus* spp.	Total *Enterococci*
Caseinase	14.73±2.40^{abcd}	15.31±8.10^{ab}	15.57±25.75	40.60±25.95^{ab}
Slime	6.98±4.41^{a}	6.39±9.50	8.01±10.90	21.38±17.85
Gelatinase	3.88±3.18^{b}	8.33±4.12	1.59±2.46	13.80±5.47^{a}
Haem-R	3.10±2.40^{c}	3.10±2.90^{a}	6.43±11.32	12.63±11.20^{b}
Haem-H	6.98±4.16^{d}	3.88±2.72^{b}	7.78±16.84	18.64±19.09

Values presented as mean ± standard deviation. Proportion of isolates positive to tested virulence assay with same letters (a–d) within column represent mean comparisons with significant differences at $p<0.05$. Haem-H and Haem-R represent heamolysis assays on horse and rabbit blood, respectively

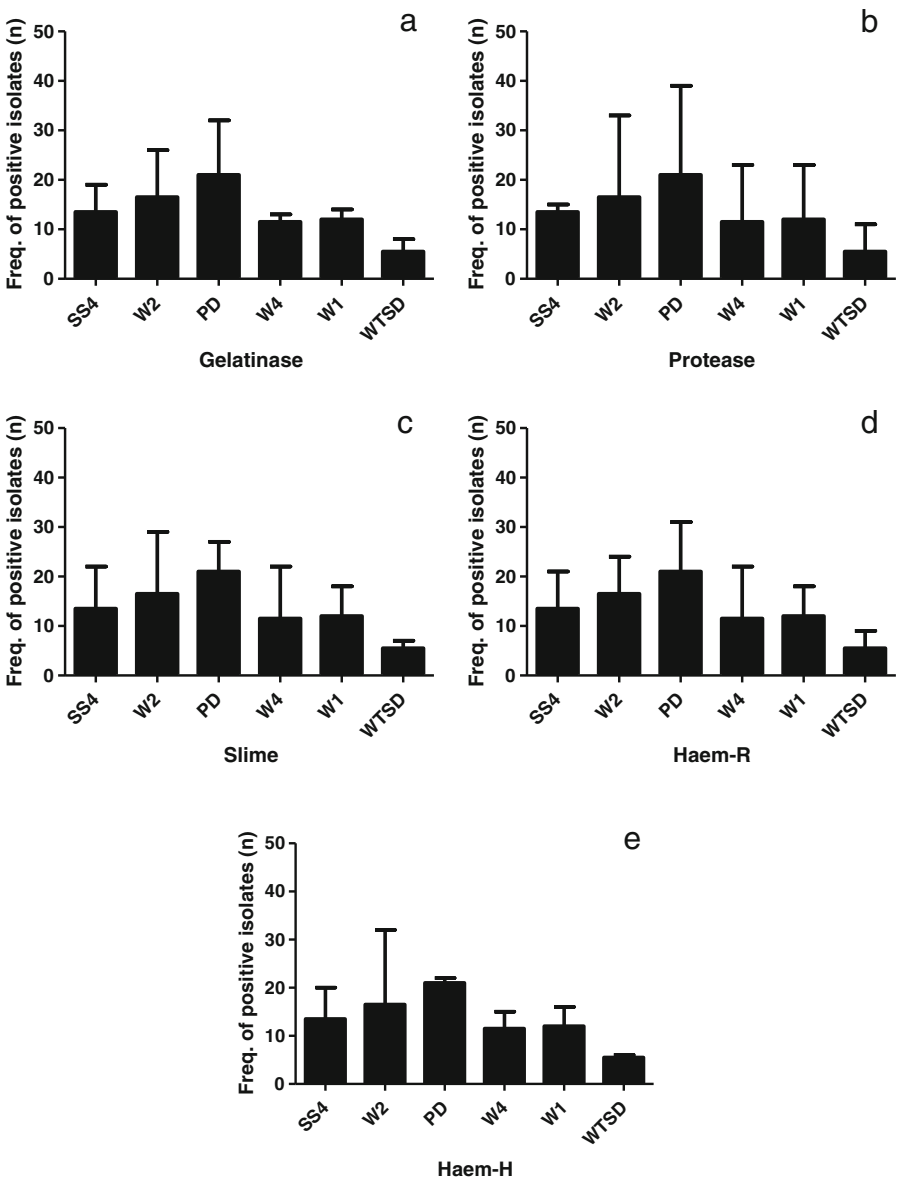

Fig. 3 **a**–**e** Source of isolation of *Enterococci* and observed virulence characteristics. Water sample collected from a neighbouring swamp about 500 m away from the beach at Bagan Lalang (*W1*). Water sample collected from the bathing area of the beach at Bagan Lalang, the area receiving visible overland flow from the environment (*WS2*). Water sample collected from the bathing area of the beach at Bagan Lalang about 500 m away from the visible overland flow from the environment but close to the massive hotels built on sea (*WS4*). Dry soil sample collected from the beach sand at Bagan Lalang (*SS4*). Wet soil sample collected from the beach sand at Bagan Lalang (*WTSD*). Water sample collected from bathing area at Teluk Kemang beach, Port Dickson (*PD*). Haem-H and Haem-R represent heamolysis assays on horse and rabbit blood, respectively

slime (C=0.50, V=0.58) assays. Analysis of obtained spearman's coefficient showed a strong correlation between caseinase and each of the slime production, gelatinase and haemolytic activity on horse blood, respectively (Table 2).

Discussion

The issue of sustainable management of coastal bathing waters remains a challenge particularly in developing nations. In these locations, uncontrollable waste discharge into seawater and other associated factors work in concert to influence the quality of water available for bathers. In previous reports, core issues affecting the bacteriological quality of recreational beaches available in Malaysia have been highlighted (Dada et al. 2012b; Praveena et al. 2011; Hamzah et al. 2011; Schwartz 2005; Thanapalasingam 2005; Aiyub 1997; Law and Othman 1990; Law et al. 2000). In addition to several wastewater discharge pipes supplying sewage from hotels and houses directly into the sea in the northern part of Port Dickson, the sea receives heavy metal pollution due to growth in tourism activities and urbanization (Praveena et al. 2011).

Table 2 Test for correlation between considered virulence characteristics among beach *Enterococci*

Virulence characteristics	Parameter	Slime	Gelatinase	Haem-R	Haem-H	Caseinase
Caseinase	Spearman *r*	0.4854	0.6496	0.4137	0.4825	N.D.
	P value (two-tailed)	0.0412	0.0035	0.0879	0.0425	
	P value summary	_[a]	_[b]	_[c]	_[a]	
Haem-H	Spearman *r*	0.2908	0.3310	0.1759	N.D.	0.4825
	P value (two-tailed)	0.2417	0.1797	0.4851		0.0425
	P value summary	_[c]	_[c]	_[c]		_[a]
Haem-R	Spearman *r*	0.5725	0.2299	N.D.	0.1759	0.4137
	P value (two-tailed)	0.0130	0.3587		0.4851	0.0879
	P value summary	_[a]	_[c]		_[c]	_[c]
Gelatinase	Spearman *r*	−0.03838	N.D.	0.2299	0.3310	0.6496
	P value (two-tailed)	0.8798		0.3587	0.1797	0.0035
	P value summary	_[c]		_[c]	_[c]	_[b]
Slime	Spearman *r*	N.D.	−0.03838	0.5725	0.2908	0.3310
	P value (two-tailed)		0.8798	0.0130	0.2417	0.1797
	P value summary		_[c]	_[a]	_[c]	_[c]

P values are based on Gaussian approximation

Haem-R haemolytic activity on rabbit blood, *Haem-H* haemolytic activity on horse blood

[a] Significant at alpha=0.05

[b] Highly significant at alpha=0.05

[c] Not significant at alpha=0.05

Chua et al. (1997) also asserts that during the last five decades, Straits of Malacca received large amounts of waste loadings from municipal, industrial, agricultural and shipping discharges. Agreeably, these discharges are ultimately to be blamed for the degradation of marine water quality.

The vulnerability of available beaches thus makes them susceptible to influx of bacteria not indigenous to the beach environment many of which may be potentially virulent or resistant to commonly used antibiotics. The presence and levels of pathogenic bacteria particularly as a result of fecal contamination of water are usually elucidated using bacteria indicators (Sinigalliano et al. 2010). The most commonly tested fecal bacteria indicators are total coliforms, fecal coliforms, *E. coli*, fecal *streptococci* and *Enterococci* (USEPA 2012). *Enterococci* have been suggested as an alternative to *E. coli* for use in recreational water surveillance due to their direct correlation to swimmer-associated gastroenteritis (Kinzelman et al. 2003). Our research group has thus focused on this genus of bacteria with particular reference to quality monitoring of coastal beach water resources in Malaysia.

It is, however, noteworthy to mention that apart from being indicator organisms, *Enterococci* are important nosocomial pathogens resistant to many popular antimicrobial agents (aminoglycosides, cephalosporins, aztreonam, semisynthetic penicillin, trimethoprim-sulphamethoxazole) (Murray 1997; Rice 2001). Generally, the levels of antibiotic resistance observed in our study were high for all the non-aminoglycoside antibiotics tested. This is in line with de Oliveira and Watanabe Pinhata (2008) who reported resistance to a larger number of antimicrobials and higher percentages of resistant strains (61.5 %) in polluted beaches as compared with a less polluted beach (31.25 %). In the current study, a high proportion of erythromycin resistance was also observed. This is of particular interest as macrolides are often used in the community for the empirical treatment of infectious diseases, as well as enterococcal infections, especially when allergic reactions to penicillins are suspected. Notably, all isolates regardless of the source of isolation were resistant to ceftriaxone. Our findings corroborate those of Gales et al. (2000) and Farina et al. (2011) who documented very high percentages of *Enterococci* as

being resistant to ceftriaxone, a long acting, broad-spectrum cephalosporin antibiotic, which exerts bactericidal activity by inhibition of cell wall synthesis.

High-level resistance to gentamicin is associated with resistance to mostly all clinically available aminoglycosides (Chow 2000). Our results indicate that the prevalence of gentamicin resistance was negligible as none of the encountered isolates were resistant to high levels of gentamicin (500 μg/mL). High-level aminoglycoside resistance in *Enterococci* is mediated generally by aminoglycoside-modifying enzymes, which eliminate the synergistic bactericidal effect usually seen when a cell wall-active agent is combined with an aminoglycoside (Chow 2000). Previously published evidence reveal that high-level gentamicin resistance is often confined to *E. faecium* (Woodford et al. 1993; McNamara et al. 1995). Papaparaskevas et al. (2000) reported a contrary observation as HLAR was confined only to *E. faecalis* in their study on the diversity of HLAR *Enterococci* in Greece. Another report observed equal rates of HLAR between the two species. In the present study, however, gentamicin resistance was not observed for either of the two species. This is in agreement with Gordon et al. (1992).

Chow (2000) argued that with the report of a number of new aminoglycoside resistance genes, there may be the need for a new approach to detecting resistance to aminoglycoside synergism. For *Enterococci* containing the new HLAR gene, aph (2″)-Ic, MIC is 256–384 μg/mL. The critical issue is that if gentamicin at 500 μg/m continues to be used to detect gentamicin resistance, isolates that possess aph (2″)-Ic may be missed and falsely deemed as susceptible. This consideration, however, was well taken care of in our study as we used a wide range of concentration (4–1,000 μg/mL), which also captures an estimated 256 μg/mL MIC for *Enterococci* that possess the gene. Another side of the argument is that since there is only a twofold dilutional difference between 256 and 500 μg/mL, strains bearing aph (2″)-Ic can at times show minimum growth in a screening test that uses 500 μg/mL gentamicin thus leading to false positives. Although no gentamicin-resistant isolate was reported in our study, a precautionary principle we would have taken had we recovered high-level gentamicin-resistant isolates was to repeat the screening using a series of stepwise dilutions that captures the specified range of contention.

Another gene encoding high-level resistance to gentamicin in *Enterococci*, aph(2″)-Ib , also reportedly mediates high-level resistance in *Enterococci* to kanamycin (Chow 2000). In our study, kanamycin resistance, albeit in a low proportion was detected in water samples collected from both beaches. It is interesting to note that resistance was higher among isolates from Port Dickson, where a number of studies have affirmed the continuous contamination of this seawater by uncontrolled sewage discharge. Owing to the uncontrolled contamination by domestic effluents reported in PD and not in BL, the observed bacterial resistance may be a result of the entrance of non-indigenous bacteria in marine ecosystems (Goni-Urriza et al. 2000). While there are no reports published on the discharge of sewage into seawater at Bagan Lalang beach, the rapid developments in this area may be potential sources of faecal contamination.

High-level aminoglycoside resistance was also detected in the beach sand. Our study is the first to isolate high-level aminoglycoside-resistant bacteria from coastal beach sand and water in Malaysia. In a previous study, the occurrence of antibiotic-resistant bacteria in beach sediment collected from Malaysia was reported (Dada et al. 2013b). A number of other studies have also reported that beach sands may act as potential reservoirs or vectors for a number of bacteria (Wheeler Alm et al. 2003; Bonilla et al. 2007; Mendes et al. 1997; Papadakis et al. 1997; Whitman and Nevers 2003). Previous studies have elucidated favourable conditions of nutrients (Davies et al. 1995; Villar et al. 1999), shelter against sunlight (Davies-Colley et al. 1999; Sinton et al. 1994) and protection against protozoan predation (Davies and Bavor 2000; Pianetti et al. 2004) as possible reasons behind the generally higher concentrations of bacteria usually encountered in sand as compared to the water column (Ghinsberg et al. 1994; Oshiro and Fujioka 1995). These factors may improve bacterial survival in sand and provide an explanation for the higher proportion of kanamycin resistance observed among sand samples as compared to the beach water samples.

In our present study, we observed that no vancomycin-resistant isolate was recovered from Bagan Lalang beach water, whereas a low level of vancomycin resistance (5.88 %) was observed among Port Dickson isolates. This observation may reflect the pollution levels of both beaches. While publications abound that document sewage pollution in PD, these are not available for Bagan

Lalang beach. The level of vancomycin resistance observed in this study, albeit in low proportions, is in concert with a previous study on recreational waters (de Oliveira et al. 2007). Kühn et al. (2000) also isolated vancomycin-resistant *Enterococci* (VRE) in superficial waters in Sweden and Spain. The treatment of vancomycin-resistant *Enterococci* is a major clinical problem since many of them are multi-drug resistant. Vancomycin resistance eliminates the synergistic activity usually achieved by aminoglycoside combination, thus leaving β-lactamase as the only choice to combine with aminoglycosides. The antibiotic of choice for such multi-drug-resistant *Enterococci* is currently not known (Adhikari 2010). While the problem of VRE may not be very high in Malaysia at present, concerted efforts at monitoring VRE is the need of the hour since it appears to be an emerging pathogen in developing economies. This study is the first to isolate and characterize VRE in recreational waters in Malaysia. Hopefully, future studies will focus on the detection of and potential transferability of bacterial genes responsible for resistance to vancomycin.

A crucial question is that which relates to the virulence of *Enterococci* isolates encountered in recreational beach water and sand samples. Are these particularly virulent? Among the several putative virulence factors in *Enterococci* are the aggregation substances (Galli et al. 1990), cytolysin (Ike et al. 1987), gelatinase (Su et al. 1991), hyaluronidase (Rice et al. 2003; Vankerckhoven et al. 2008) and enterococcal surface protein (Shankar et al. 1999; Rosa et al. 2006). A number of reports affirm that *Enterococci* with the highest virulence are medical isolates, followed by food isolates and then starter strains (Busani et al. 2004; Omar et al. 2004; Fisher and Phillips 2009). Notably missing in these studies are the environmental strains. Over the years, most researches have focused mainly on clinical isolates with the assumption of higher public health threats mediated by these isolates. However, as argued in a previous report, such clearly cut lines of an isolate being of a clinical source or environmental source may remain more or less definitional concepts (Dada et al. 2012a). This is particularly so because improper waste management systems in developing countries may warrant the discovery in environmental settings, isolates with alarming levels of antibiotic resistance and virulent phenotypes noticeable in laboratory experiments. Even with the increasing recognition of the clinical importance of enterococcal infections, their virulence mechanisms are still not well understood (Sifri et al. 2002).

In the present study, we screened a total of 160 beach *Enterococci* isolates for virulence characteristics. On an inter-species basis, the percentage of strains demonstrating virulence characteristics in the current study was observably highest among the isolates other than *E. faecalis* and *E. faecium*, herein referred to as *Enterococci* spp. Trivedi et al. (2011) also reported the occurrence of virulence genes among species other than *E. faecalis* and *E. faecium*. These findings differ from those of Vankerckhoven et al. (2004) and Martín-Platero et al. (2009) who observed the presence of similar virulence traits in *E. faecalis* and *E. faecium* only. Isolates other than *E. faecalis* and *E. faecium* demonstrated the highest levels of virulence in the present study. Although in clinical settings, *E. faecalis* and *E. faecium* are reportedly responsible for up to 95 % of enterococcal infections, some of the less pathogenic *Enterococci* (other than *E. faecalis* and *E. faecium*) have also been isolated from serious infections (Kurup et al. 2001; Iaria et al. 2005).

Cytolysin is one of the molecules secreted by *Enterococci* as a putative virulence factor that shows haemolytic activity (against human, horse, and rabbit erythrocytes) and bactericidal effects on other Gram-positive bacteria (Coque et al. 1995). We initially tested all the 160 isolates of beach *Enterococci* for haemolytic activities using sheep blood supplemented agar. Interestingly, none were haemolytic. This explains why additional erythrocyte types were used in the present study, particularly horse and rabbit. Similarly, Miyazaki et al. (1993) reported that BHI culture supernatants without additional nutritional supplements did not show haemolytic activity on sheep blood suggesting that glucose and arginine may be essential elements for heat stable haemolysin production. Our study is in agreement with those of Miyazaki et al. (1993) who detected haemolytic activity on BHIA supplemented with several animal erythrocytes apart from cow and sheep.

A number of studies have highlighted the importance of haemolysin in enterococcal virulence, particularly in increasing the severity of the infection (Huycke et al. 1991; Jett et al. 1994). Tsikrikonis et al. (2012) reported a higher proportion of clinical *E. faecalis* strains producing haemolysin as compared to *E. faecium*. In another study by Ike et al. (1987), up to

60 % of clinical *E. faecalis* isolates demonstrated haemolytic activity as compared to 17 % of strains from uninfected sources. The incidence of hemolysin in our study was lower than was reported in another study by Eaton and Gasson (2001). Remarkably, for haemolysis of rabbit and horse blood, prevalence of isolates demonstrating this phenotype was low among both *E. faecium* and *E. faecalis*. While no significant difference was observed for the haemolysis of rabbit blood among *E. faecium and E. faecalis*, haemolysis of horse blood was more profound among the *E. faecalis* isolates in our study.

On the whole, the least prevalent virulence characteristics observed was haemolysis of rabbit blood. The proportion of beta haemolytic isolates observed in our study is low as compared to the very high proportions (75 %) of haemolytic *Enterococci* reported by Furumura et al. (2006). Those isolates, however, were clinical strains as opposed to the environmental strains considered in our study. In the present study, beta-haemolytic behavior on horse blood was appreciably low. However, an analysis of beta-haemolytic behavior among *Enterococci* spp. showed a higher prevalence in species other than *E. faecalis* and *E. faecium*. This is contrary to the findings of previous reports (Jett et al. 1994; Johnson 1994; Mundy et al. 2000; Semedo et al. 2003). In our study, there was a significant association between haemolytic activity on horse blood and species allocation of isolates (p=0.0256). The absence of significant association between haemolytic activity on rabbit blood and species allocation may not possibly be due to sample size effect but instead a species-specific preference for horse blood. The reason for this needs to be substantiated in future studies. Also, future studies would extend beyond mere detection of in vivo cytolytic activities to implicating specific beach enterococcal virulence factors in clinical mortality.

Among the virulence assays conducted, caseinase activity was notably the highest across all species of *Enterococci* tested. While generally grouped as being a protease together with gelatinase, there appears to be a paucity of scholarly discussion on caseinase. Nevertheless, its role in the breakdown of host tissues for the purpose of survival and establishment in the host are crucial to the organism. Furumura et al. (2006) suggests based on data obtained that gelatinase hydrolysing activity is different from caseinase although definitional linkages bind them. The same observation was made in our present study as prevalence of gelatinase activity among *Enterococci* isolates was significantly different from that of caseinase.

However, it is noteworthy to mention that Spearman's analysis revealed a strong correlation between caseinase and gelatinase. Gelatinase, is an extracellular metalloprotease reportedly secreted by *E. faecalis*, hyrdolyses gelatin, collagen and casein, and has been implicated as a virulence factor in animal models (Furumura et al. 2006). Waters et al. (2003) also iterated the potential ability of this enzyme to hyrdolyse bioactive peptides especially in the initiation and propagation of inflammatory processes by the bacteria. Semedo et al. (2003), however, argues that the precise functions of gelatinase and other hydrolytic enzymes are still poorly understood. For instance, isogenic strains of *E. faecalis* differing in gelatinase production appear only to modestly affect acute toxicity in the bolus LD50 murine model.

Notwithstanding, Vergis et al. (2002) demonstrated that 64 % of *E. faecalis* isolated from patients with bacteremia, produced gelatinase. In another study, protease-producing *E. faecalis* was prevalent among *Enterococci* with isolation rates of as high as 63.7 % in a clinical setting (Kühnen et al. 1988). More than half of the 95 enterococcal isolates from patients with endocarditis and other nosocomial infections were found to produce protease in the report published by Coque et al. (1995). A low proportion (12 %) of enterococcal isolates was, however, recorded for caseinase positive isolates recovered from uninfected individuals. This reported proportion is lower than the observed proportion of gelatinase positive *Enterococci* (40.40 %) in our study.

While gelatinase activity is thought to be exclusive to *E. faecalis* and not *E. faecium*, reports vary in their position on the species prevalence of this virulence factor (Mohamed and Huang 2007). In a study by Tsikrikonis et al. (2012), none of the clinical *E. faecium* strains and 34.4 % of clinical *E. faecalis* strains were found to produce gelatinase. While Kanemitsu et al. (2001) and Duprè et al. (2003) both reported no gelatinase activity in all *E. faecium* tested, Lhan et al. (2006) reported that GelE gene was detected in similar proportions among *E. faecium* and *E. faecalis* isolates. Macovei et al. (2009) reported gelatinase activity among 25.9 % of *Enterococcus faecium* isolates recovered in a cattle farm setting. In our study, 3.87 % and 8.3 % respectively of *E. faecalis and E. faecium* populations recovered from the considered beaches

hydrolysed gelatin. While these proportions observed are fairly low as compared to clinical *Enterococci*, notable is the fact that *E. faecium* isolates in the present study demonstrated even higher gelatinase activity than *E. faecalis*, which is most commonly reported to demonstrate the virulence characteristics. Gelatinase and serine protease (SprE) in *E. faecalis* are encoded in an operon, *gelE–sprE*, whose expression is positively regulated by a quorum sensing system encoded by the *fsr* locus (Qin et al. 2000). Findings by Lopes et al. (2006) in a study on diary *Enterococci* elucidated that gelatinase production may be underestimated for non-clinical isolates and in non-*E. faecalis* species. The study published by Lopes et al. (2006) was the first that reported the detection of the complete Fsr-GelE operon in other species than *E. faecalis,* namely *E. faecium* and *E. durans*. The report concluded that gelatinase genetic determinants, so far only described in *E. faecalis*, are a common trait in the entire genus. This arguably justifies the observed variance in our present study.

Previous studies have also highlighted functional capabilities of proteases particularly in biofilm formation (Gilmore 2002; Mohamed and Huang 2007). It was thus not surprising that Spearman's analysis revealed strong correlations between these caseinase and slime production. The ability of *Enterococci* to produce biofilms is fundamental in causing endodontic and urinary tract infections, as well as endocarditis. Together with other factors, biofilm production makes it possible for the bacteria to adhere and colonize the host tissue (Mundy et al. 2000; Baldassarri et al. 2001a, b). The importance of this virulence factor was noted in our study as it was the second most prevalent virulence characteristics observed among all species of tested beach *Enterococci*. As was also observed in our study, similar rates of *E. faecalis* and *E. faecium* isolates had capacity for biofilm formation in a recent study by Tsikrikonis et al. (2012). Baldassarri et al. (2001a) reported biofilm production in 80 % of *E. faecalis* and 48 % of *E. faecium* isolates from infected patients. Another study (Duprè et al. 2003) in the same country reported 87 % and 16 %, respectively of biofilm producing *E. faecalis* and *E. faecium* clinical isolates. While there are variances in reported prevalence of biofilm production among clinical isolates (Mohamed and Huang 2007), such comparisons abound mostly for clinical isolates and faecal human or animal isolates. There is a paucity of information on comparisons between clinical and environmental strains, particularly those recovered from recreational beaches.

We also tested all isolates for lipase production. None was positive to the lipase assays conducted using egg yolk supplemented agar. It appears not to be an important virulence factor for the genus *Enterococci*. Moreover, studies that relate *Enterococci* to lipase production are scarce in literature (Furumura et al. 2006). More studies are needed that elucidate the role of lipases in Enterococcal infections. Substantiation analysis of associations between the entire virulence characteristics tested in this study using Cramers statistics revealed a strong association between the source of isolation and responses for each of caseinase and slime assays. Furthermore, spearman's correlation coefficient analysis showed strong correlations between caseinase and each of slime production, gelatinase and horse blood haemolysis. Understandably, these have to do with attachment to and breaking down of binding tissues of host/reservoirs in their bid for survival and or establishment. We thus hypothesize that the main virulent properties of beach *Enterococci* isolated from Malaysia are the formation of biofilm, followed by breakdown of host tissues by caseinases ultimately leading to the formation of haemolysin, and possibly other virulence factors which define the extent of pathogenicity that may be observable in clinical cases caused by this organism. This is the first study that provides new insight in this direction among non-clinical isolates and particularly among beach-encountered *Enterococci* in Malaysia.

Conclusion

Our findings suggest that recreational beaches may contribute to the dissemination of HLAR *Enterococci* associated with community infections. The successful isolation of HLAR *Enterococci* in environmental samples, albeit in low proportions may warrant concerns both for clinicians and relevant heath authorities. Our study revealed strong correlations between caseinase and each of the slime production, gelatinase and horse blood haemolytic activity. Suggestively, these are the main virulence characteristics of the encountered beach *Enterococci*. A potential limitation to the current study, however, is that it does not focus on particular clones or mobile elements carrying HLAR or virulence traits as suggested by Novais et al. (2005). This may present a clearer picture linking the contamination of these

recreational beaches by uncontrolled discharge of wastewater with HLAR or virulence characteristics observable in our study. It is hoped that future studies will emerge that will fulfill these criteria. Nonetheless, critical is the need for concerted efforts aimed at reducing the indiscriminate release of bacteria-laden waste into the coastal environment. Ultimately, this will prevent the build-up of environmental reservoirs of high-level antibiotic resistance and/or virulence characteristics.

Acknowledgments Obafemi Awolowo University, Ile-Ife, Nigeria gratefully granted study leave for the research.

Financial support Universiti Kebangsaan Malaysia Marine Pathogen Program Grant under the auspices of the School of Bioscience and Biotechnology, Faculty of Science and Technology.

References

Adhikari, L. (2010). High-level aminoglycoside resistance and reduced susceptibility to vancomycin in nosocomial Enterococci. *Journal of Global Infectious Diseases, 2*(3), 231.

Aiyub, K. (1997). Utilization and management of the Negeri Sembilan coastal zone. *Akademika* (50), 3–23.

American Public Health Association (APHA) (1999). Standard methods for the examination of waters and wastewaters (20th ed.) APHA, Washington, DC, USA.

Anonymous. (2011). Welcome to Port Dickson: Popular holiday destination and camping site in Peninsular Malaysia. Available at http://www.port-dickson.net/2010/02/teluk-kemang-beach/. Accessed 10 October 2011.

Archimbaud, C., Shankar, N., Forestier, C., Baghdayan, A., Gilmore, M. S., Charbonné, F., et al. (2002). In vitro adhesive properties and virulence factors of Enterococcus faecalis strains. *Research in microbiology, 153*(2), 75–80.

Arciola, C. R., Baldassarri, L., Montanaro, L. (2002). In catheter infections by *Staphylococcus epidermidis* the intercellular adhesion (ica) locus is a molecular marker of the virulent slime-producing strains. *Journal of Biomedical Materials Research, 59*, 557–562.

Arias, C. A., & Karchmer, A. W. (2011). Prevention of antimicrobial resistance current and future strategies. In A. Safdar (Ed.), *Principles and Practice of Cancer Infectious Diseases* (pp 507–517). Humana Press, c/o Springer Science+Business Media, LLC, 233 Spring Street, New York, NY 10013, USA.

Baldassarri, L., Bertuccini, L., Ammendolia, M. G., Gherardi, G., & Creti, R. (2001a). Variant esp gene as a marker of a distinct genetic lineage of vancomycin-resistant *Enterococcus faecium*. *The Lancet, 357*(9270), 1802.

Baldassarri, L., Cecchini, R., Bertuccini, L., Ammendolia, M. G., Iosi, F., Arciola, C. R., et al. (2001b). *Enterococcus* spp. produces slime and survives in rat peritoneal macrophages. *Medical Microbiology and Immunology, 190*(3), 113–120.

Bonilla, T. D., Nowosielski, K., Cuvelier, M., Hartz, A., Green, M., Esiobu, N., et al. (2007). Prevalence and distribution of fecal indicator organisms in South Florida beach sand and preliminary assessment of health effects associated with beach sand exposure. *Marine Pollution Bulletin, 54*(9), 1472–1482.

Brenden, R., & Janda, J. (1987). Detection, quantitation and stability of the β haemolysin of Aeromonas spp. *Journal of Medical Microbiology, 24*(3), 247–251.

Busani, L., Del Grosso, M., Paladini, C., Graziani, C., Pantosti, A., Biavasco, F., et al. (2004). Antimicrobial susceptibility of vancomycin-susceptible and -resistant Enterococci isolated in Italy from raw meat products, farm animals, and human infections. *International Journal of Food Microbiology, 97*(1), 17–22.

Cariolato, D., Andrighetto, C., & Lombardi, A. (2008). Occurrence of virulence factors and antibiotic resistances in *Enterococcus faecalis* and *Enterococcus faecium* collected from dairy and human samples in North Italy. *Food Control, 19*(9), 886–892.

Casas, V., Sobrepeña, G., Rodriguez-Mueller, B., AhTye, J., & Maloy, S. R. (2011). Bacteriophage-encoded shiga toxin gene in atypical bacterial host. *Gut Pathogens, 3*(1), 1–7.

Chow, J. W. (2000). Aminoglycoside resistance in Enterococci. *Clinical Infectious Diseases, 31*(2), 586–589.

Chua, T. E., Ross, S. A., & Yu, H. (1997). Malacca straits environmental profile. In B. J. Sidik, A. Arshad, M. H. Zakaria, & A. Kawamura (Eds.), *Aquatic resource and environmental studies of the straits of Malacca*. Serdang: University of Putra Malaysia.

Clinical and Laboratory Standards Institute (CLSI) (2008). Performance standards for antimicrobial susceptibility testing; eighteenth informational supplement, M100-S18CLSI, Wayne, PA.

Coque, T. M., Patterson, J. E., Steckelberg, J. M., & Murray, B. E. (1995). Incidence of hemolysin, gelatinase, and aggregation substance among Enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. *Journal of Infectious Diseases, 171*(5), 1223.

Cosgrove, S. E. (2006). The relationship between antimicrobial resistance and patient outcomes: mortality, length of hospital stay, and health care costs. *Clinical Infectious Diseases, 42*(Supplement 2), S82–S89.

Crewson, P. (2006). Applied statistics handbook on *AcaStat Software*, pp. 1–12. Available at http://www.isibang.ac.in/~library/onlinerz/resources/Stathandbookexample.pdf. Accessed 7 June 2012.

Dada, A. C., Ahmad, A., Usup, G., & Heng, L. Y. (2013a). Confirmation of putative storm water input at Teluk Kemang, Port Dickson. *Advances in Environmental Biology*, in press.

Dada, A. C., Ahmad, A., Usup, G., & Heng, L. Y. (2013b). Speciation and antimicrobial resistance of *Enterococci* isolated from recreational beaches in Malaysia. *Environmental Monitoring and Assessment, 185*(2), 1583–1599.

Dada, A. C., Ahmad, A., Usup, G., & Heng, L. Y. (2012a). Antibiotic resistance and virulence among *Enterococci* isolated from Teluk Kemang Beach, Malaysia. *Water Quality, Exposure and Health*, 1–10. doi:10.1007/s12403-012-0070-2.

Dada, A. C., Asmat, A., Gires, U., Heng, L. Y., & Deborah, B. O. (2012b). Bacteriological monitoring and sustainable

management of beach water quality in Malaysia: problems and prospects. *Global Journal of Health Science, 4*(3), 126. doi:10.5539/gjhs.v4n3p126.

Dang, H., Ren, J., Song, L., Sun, S., & An, L. (2008). Dominant chloramphenicol-resistant bacteria and resistance genes in coastal marine waters of Jiaozhou Bay, China. *World Journal of Microbiology and Biotechnology, 24*(2), 209–217.

Davies, C., & Bavor, H. (2000). The fate of storm water-associated bacteria in constructed wetland and water pollution control pond systems. *Journal of Applied Microbiology, 89*(2), 349–360.

Davies, C. M., Long, J., Donald, M., & Ashbolt, N. J. (1995). Survival of fecal microorganisms in marine and freshwater sediments. *Applied and Environmental Microbiology, 61*(5), 1888–1896.

Davies-Colley, R., Donnison, A., Speed, D., Ross, C., & Nagels, J. (1999). Inactivation of faecal indicator micro-organisms in waste stabilisation ponds: interactions of environmental factors with sunlight. *Water Research, 33*(5), 1220–1230.

de Oliveira, A. J. F. C., & Watanabe Pinhata, J. M. (2008). Antimicrobial resistance and species composition of *Enterococcus* spp. isolated from waters and sands of marine recreational beaches in Southeastern Brazil. *Water Research, 42*(8–9), 2242–2250.

de Oliveira, A. J. F. C., Hollnagel, H. C., Lima Mesquita, H. S., & Fontes, R. F. C. (2007). Physical, chemical and microbiological characterization of the intertidal sediments of Pereque Beach, Guarujá (SP), Brazil. *Marine Pollution Bulletin, 54*(7), 921–927.

Debacker, V., Jauniaux, T., Coignoul, F., & Bouquegneau, J. M. (2000). Heavy metals contamination and body condition of wintering guillemots (*Uria aalge*) at the Belgian coast from 1993 to 1998. *Environmental Research, 84*(3), 310–317.

Department of Environmental Malaysia, DOE (2006). Malaysia environmental quality report 2006 in Impak, Kuala Lumpur: Ministry of Science. *Technology and Environment, 3*, 1–16.

Duprè, I., Zanetti, S., Schito, A. M., Fadda, G., & Sechi, L. A. (2003). Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). *Journal of Medical Microbiology, 52*(6), 491–498.

Eaton, T. J., & Gasson, M. J. (2001). Molecular screening of Enterococcus virulence determinants and potential for genetic exchange between food and medical isolates. *Applied and Environmental Microbiology, 67*(4), 1628–1635.

Facklam, R. (2002). What happened to the streptococci: overview of taxonomic and nomenclature changes. [Review]. *Clinical Microbiology Reviews, 15*(4), 613–630. doi:10.1128/cmr.15.4.613-630.2002.

Facklam, R., & Collins, M. (1989). Identification of Enterococcus species isolated from human infections by a conventional test scheme. *Journal of clinical microbiology, 27*(4), 731–734.

Facklam, R., & Elliott, J. A. (1995). Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clinical Microbiology Reviews, 8*(4), 479.

Farina, C., Russello, G., Chinello, P., Pasticci, M., Raglio, A., Ravasio, V., et al. (2011). In vitro activity effects of twelve antibiotics alone and in association against twenty-seven *Enterococcus faecalis* strains isolated from Italian patients with infective endocarditis: High in vitro synergistic effect of the association ceftriaxone-fosfomycin. *Chemotherapy, 57*(5), 426–433.

Fernandez, L., Breidenstein, E. B. M., & Hancock, R. E. W. (2011). Importance of adaptive and stepwise changes in the rise and spread of antimicrobial resistance. In P. L. Keen & M. H. M. M. Montforts (Eds.), Antimicrobial Resistance in the Environment (pp. 43–71), John Wiley & Sons Inc., Hoboken, New Jersey.

Fisher, K., & Phillips, C. (2009). The ecology, epidemiology and virulence of Enterococcus. *Microbiology, 155*(6), 1749–1757.

Fraimow, H. S., & Tsigrelis, C. (2011). Antimicrobial resistance in the intensive care unit: mechanisms, epidemiology, and management of specific resistant pathogens. *Critical Care Clinics, 27*(1), 163.

Furumura, M. T., Figueiredo, P., Carbonell, G. V., Darini, A. L. C., & Yano, T. (2006). Virulence-associated characteristics of *Enterococcus faecalis* strains isolated from clinical sources. *Brazilian Journal of Microbiology, 37*(3), 230–236.

Gales, A. C., Jones, R. N., Gordon, K. A., Sader, H. S., Wilke, W. W., Beach, M. L., et al. (2000). Activity and spectrum of 22 antimicrobial agents tested against urinary tract infection pathogens in hospitalized patients in Latin America: report from the second year of the SENTRY antimicrobial surveillance program (1998). *Journal of Antimicrobial Chemotherapy, 45*(3), 295–303.

Galli, D., Lottspeich, F., & Wirth, R. (1990). Sequence analysis of *Enterococcus faecalis* aggregation substance encoded by the sex pheromone plasmid pAD1. *Molecular Microbiology, 4*(6), 895–904.

Ghinsberg, R., Bar, D. L., Rogol, M., Sheinberg, Y., & Nitzan, Y. (1994). Monitoring of selected bacteria and fungi in sand and sea water along the Tel Aviv coast. *Microbios, 77*(310), 29.

Gilmore, M. S. (2002). *The enterococci: pathogenesis, molecular biology, and antibiotic resistance*. Washington, DC: American Society for Microbiology (ASM) Press.

Goni-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Caumette, P., & Quentin, C. (2000). Impact of an urban effluent on antibiotic resistance of riverine *Enterobacteriaceae* and *Aeromonas* spp. *Applied and Environmental Microbiology, 66*(1), 125–132.

Gordon, S., Swenson, J. M., Hill, B., Pigott, N., Facklam, R., Cooksey, R., et al. (1992). Antimicrobial susceptibility patterns of common and unusual species of Enterococci causing infections in the United States. Enterococcal Study Group. *Journal of clinical microbiology, 30*(9), 2373–2378.

Graham, J. L., Loftin, K. A., & Kamman, N. (2009). Monitoring recreational freshwaters. *Lakelines, 29*, 18–24.

Grant, S. B., Sanders, B., Boehm, A., Redman, J., Kim, J., Mrše, R., et al. (2001). Generation of Enterococci bacteria in a coastal saltwater marsh and its impact on surf zone water quality. *Environmental Science & Technology, 35*(12), 2407–2416.

Guzmán, H. M., & Jiménez, C. E. (1992). Contamination of coral reefs by heavy metals along the Caribbean coast of

Central America (Costa Rica and Panama). *Marine Pollution Bulletin, 24*(11), 554–561.

Hamzah, A., Kipli, S. H., & Rahil Ismail, S. I. I. (2011). Microbiological study in coastal water of Port Dickson, Malaysia. *Sains Malaysiana, 40*(2), 93–99.

Hatcher, J., Dhillon, R., & Azadian, B. (2012). Antibiotic resistance mechanisms in the intensive care unit 1A02 2C03 3A13.

Huycke, M., Spiegel, C., & Gilmore, M. (1991). Bacteremia caused by haemolytic, high-level gentamicin-resistant *Enterococcus faecalis*. *Antimicrobial Agents and Chemotherapy, 35*(8), 1626–1634.

Iaria, C., Stassi, G., Costa, G., Di Leo, R., Toscano, A., & Cascio, A. (2005). Enterococcal meningitis caused by *Enterococcus casseliflavus*. First case report. *BMC Infectious Diseases, 5*(1), 3.

Ike, Y., Hashimoto, H., & Clewell, D. B. (1987). High incidence of hemolysin production by *Enterococcus (Streptococcus) faecalis* strains associated with human parenteral infections. *Journal of Clinical Microbiology, 25*, 1524–1528.

James, G. (2010). Universal bacterial identification by PCR and DNA sequencing of 16S rRNA gene. In I. W. J. Carter, M. Schuller, G. S. James, T. P. Sloots, & C. L. Halliday (Eds.), *PCR for Clinical Microbiology An Australian and International Perspective* (pp. 209–214). New York: Springer.

Jett, B. D., Huycke, M. M., & Gilmore, M. S. (1994). Virulence of Enterococci. *Clinical Microbiology Reviews, 7*(4), 462–478.

Johnson, A. P. (1994). The pathogenicity of Enterococci. *Journal of Antimicrobial Chemotherapy, 33*(6), 1083–1089.

Kanemitsu, K., Nishino, T., Kunishima, H., Okamura, N., Takemura, H., Yamamoto, H., et al. (2001). Quantitative determination of gelatinase activity among Enterococci. *Journal of Microbiological Methods, 47*(1), 11–16.

Kinzelman, J., Ng, C., Jackson, E., Gradus, S., & Bagley, R. (2003). Enterococci as indicators of Lake Michigan recreational water quality: comparison of two methodologies and their impacts on public health regulatory events. *Applied and Environmental Microbiology, 69*(1), 92–96.

Kouidhi, B., Zmantar, T., Mahdouani, K., Hentati, H., & Bakhrouf, A. (2011). Antibiotic resistance and adhesion properties of oral Enterococci associated to dental caries. *BMC microbiology, 11*(1), 155.

Kühn, I., Iversen, A., Burman, L., Olsson-Liljequist, B., Franklin, A., Finn, M., et al. (2000). Epidemiology and ecology of Enterococci, with special reference to antibiotic resistant strains, in animals, humans and the environment: example of an ongoing project within the European research programme* This ongoing project forms a part of the 4th framework of the European research programme for Agriculture and Fisheries (FAIR). The project started in January 1998, and will be finished in June 2001.*. *International Journal of Antimicrobial Agents, 14*(4), 337–342.

Kühnen, E., Richter, F., Richter, K., & Andries, L. (1988). Establishment of a typing system for group D streptococci. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. Series A: Medical Microbiology, Infectious Diseases, Virology, Parasitology, 267*(3), 322–330.

Kurup, A., Tee, W. S. N., Loo, L. H., & Lin, R. (2001). Infection of central nervous system by motile *Enterococcus*: first case report. *Journal of Clinical Microbiology, 39*(2), 820–822.

Kuylenstierna, J. L., Björklund, G., & Najlis, P. (2009). Sustainable water future with global implications: everyone's responsibility. *Natural Resources Forum, 21*, 181–190.

Kwon, K. H., Hwang, S. Y., Moon, B. Y., Park, Y. K., Shin, S., Hwang, C. Y., et al. (2012). Occurrence of antimicrobial resistance and virulence genes, and distribution of enterococcal clonal complex 17 from animals and human beings in Korea. *Journal of Veterinary Diagnostic Investigation, 24*(5), 924–931.

Law, A., & Othman, A. (1990). Sewage pollution in the coastal waters off Port Dickson, Straits of Malacca. *Pertanika, 13*, 375–380.

Law, A., Abdul-Rashid, M., Tan, C., & Yuen, Y. (2000). Malacca straits expedition cruise no. 1: nitrogen and phosphorus distribution from Langkawi to Johore. In: *Proceedings of International Conference on the Straits of Malacca*, April 19–22, 2000, Malacca, Malaysia, pp. 19–22.

Lhan, T. G., Akksakal, A., & Ekun, U. H. (2006). Virulence factors of *Enterococcus faecium* and *Enterococcus faecalis* strains isolated from humans and pets. *Turkish Journal of Veterinary and Animal, 30*, 477–482.

Lopes, M. F. S., Simões, A. P., Tenreiro, R., Marques, J. J. F., & Crespo, M. T. B. (2006). Activity and expression of a virulence factor, gelatinase, in dairy Enterococci. *International Journal of Food Microbiology, 112*(3), 208–214.

Louie, M., Simor, A., Szeto, S., Patel, M., Kreiswirth, B., & Low, D. (1992). Susceptibility testing of clinical isolates of *Enterococcus faecium* and *Enterococcus faecalis*. *Journal of Clinical Microbiology, 30*(1), 41–45.

Macovei, L., Ghosh, A., Thomas, V. C., Hancock, L. E., Mahmood, S., & Zurek, L. (2009). *Enterococcus faecalis* with the gelatinase phenotype regulated by the fsr operon and with biofilm-forming capacity are common in the agricultural environment. *Environmental Microbiology, 11*(6), 1540–1547.

Martín-Platero, A. M., Valdivia, E., Maqueda, M., & Martínez-Bueno, M. (2009). Characterization and safety evaluation of Enterococci isolated from Spanish goats' milk cheeses. *International Journal of Food Microbiology, 132*(1), 24–32.

McLellan, S. L., Hollis, E. J., Depas, M. M., Van Dyke, M., Harris, J., & Scopel, C. O. (2007). Distribution and fate of *Escherichia coli* in Lake Michigan following contamination with urban stormwater and combined sewer overflows. *Journal of Great Lakes Research, 33*(3), 566–580.

McNamara, E., King, E., & Smyth, E. (1995). A survey of antimicrobial susceptibility of clinical isolates of *Enterococcus* spp. from Irish hospitals. *Journal of Antimicrobial Chemotherapy, 35*(1), 185–189.

Mendes, B., Urbano, P., Alves, C., Lapa, N., Morais, J., Nascimento, J., et al. (1997). Sanitary quality of sands from beaches of Azores islands. *Water Science and Technology, 35*(11), 147–150.

Mendiratta, D. K., Kaur, H., Deotale, V., Thamke, D. C., Narang, R., & Narang, P. (2008). Status of high level

aminoglycoside resistant *Enterococcus faecium* and *Enterococcus faecalis* in a rural hospital of central India. *Indian Journal of Medical Microbiology, 26*, 369–371.

Miyazaki, S., Ohno, A., Kobayashi, I., Uji, T., Yamaguchi, K., & Goto, S. (1993). Cytotoxic effect of haemolytic culture supernatant from *Enterococcus faecalis* on mouse polymorphonuclear neutrophils and macrophages. *Microbiology and Immunology, 37*(4), 265.

Mohamed, J. A., & Huang, D. B. (2007). Biofilm formation by Enterococci. *Journal of Medical Microbiology, 56*(12), 1581–1588.

Mundy, L., Sahm, D., & Gilmore, M. (2000). Relationships between enterococcal virulence and antimicrobial resistance. *Clinical Microbiology Reviews, 13*(4), 513–522.

Murray, B. E. (1997). Vancomycin-resistant Enterococci. *The American Journal of Medicine, 102*(3), 284–293.

Novais, C., Coque, T. M., Ferreira, H., Sousa, J. C., & Peixe, L. (2005). Environmental contamination with vancomycin-resistant Enterococci from hospital sewage in Portugal. *Applied and Environmental Microbiology, 71*(6), 3364–3368.

Olapade, O. A., Depas, M. M., Jensen, E. T., & McLellan, S. L. (2006). Microbial communities and fecal indicator bacteria associated with Cladophora mats on beach sites along Lake Michigan shores. *Applied and Environmental Microbiology, 72*(3), 1932–1938.

Omar, N. B., Castro, A., Lucas, R., Abriouel, H., Yousif, N. M. K., Franz, C. M. A. P., et al. (2004). Functional and safety aspects of Enterococci isolated from different Spanish foods. *Systematic and Applied Microbiology, 27*(1), 118–130.

Ongley, E. D. (1997). Matching water quality programmes to management needs in developing countries: the challenge of programme modernization. *European Water Pollution Control, 7*(4), 43–48.

Oshiro, R., & Fujioka, R. (1995). Sand, soil, and pigeon droppings: sources of indicator bacteria in the waters of Hanauma Bay, Oahu, Hawaii. *Water Science and Technology, 31*(5), 251–254.

Papadakis, J., Mavridou, A., Richardson, S., Lampiri, M., & Marcelou, U. (1997). Bather-related microbial and yeast populations in sand and seawater. *Water Research, 31*(4), 799–804.

Papaparaskevas, J., Vatopoulos, A., Tassios, P., Avlami, A., Legakis, N., & Kalapothaki, V. (2000). Diversity among high-level aminoglycoside-resistant Enterococci. *Journal of Antimicrobial Chemotherapy, 45*(3), 277–283.

Pianetti, A., Bruscolini, F., Sabatini, L., & Colantoni, P. (2004). Microbial characteristics of marine sediments in bathing area along Pesaro-Gabicce coast (Italy): a preliminary study. *Journal of Applied Microbiology, 97*(4), 682–689.

Praveena, S. M., Siraj, S. S., Suleiman, A. K., & Aris, A. Z. (2011). A brush up on water quality studies of Port Dickson, Malaysia. *Research Journal of Environmental Sciences, 5*(12), 841–849.

Qin, X., Singh, K. V., Weinstock, G. M., & Murray, B. E. (2000). Effects of *Enterococcus faecalis* fsr genes on production of gelatinase and a serine protease and virulence. *Infection and Immunity, 68*(5), 2579–2586.

Rice, L. B. (2001). Emergence of vancomycin-resistant Enterococci. *Emerging Infectious Diseases, 7*(2), 183.

Rice, L. B., Carias, L., Rudin, S., Vael, C., Goossens, H., Konstabel, C., et al. (2003). A potential virulence gene, hylEfm, predominates in *Enterococcus faecium* of clinical origin. *Journal of Infectious Diseases, 187*(3), 508–512.

Rosa, R., Creti, R., Venditti, M., D'Amelio, R., Arciola, C. R., Montanaro, L., et al. (2006). Relationship between biofilm formation, the enterococcal surface protein (Esp) and gelatinase in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium*. *FEMS Microbiology Letters, 256*(1), 145–150.

Sahm, D., Boonlayangoor, S., & Schulz, J. (1991). Detection of high-level aminoglycoside resistance in Enterococci other than *Enterococcus faecalis*. *Journal of Clinical Microbiology, 29*(11), 2595–2598.

Schwartz, M. L. (2005). *Encyclopedia of coastal science (vol. 24)* (p. 981). Netherlands: Springer.

Schwartz, T., Kohnen, W., Jansen, B., & Obst, U. (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiology Ecology, 43*(3), 325–335.

Semedo, T., Santos, M. A., Martins, P., Lopes, M. F. S., Marques, J. J. F., Tenreiro, R., et al. (2003). Comparative study using type strains and clinical and food isolates to examine haemolytic activity and occurrence of the cyl operon in Enterococci. *Journal of Clinical Microbiology, 41*(6), 2569–2576.

Shankar, V., Baghdayan, A. S., Huycke, M. M., Lindahl, G., & Gilmore, M. S. (1999). Infection-derived *Enterococcus faecalis* strains are enriched in esp, a gene encoding a novel surface protein. *Infection and Immunity, 67*(1), 193–200.

Sifri, C. D., Mylonakis, E., Singh, K. V., Qin, X., Garsin, D. A., Murray, B. E., et al. (2002). Virulence effect of *Enterococcus faecalis* protease genes and the quorum-sensing locus fsr in *Caenorhabditis elegans* and mice. *Infection and Immunity, 70*(10), 5647–5650.

Sinigalliano, C. D., Fleisher, J. M., Gidley, M. L., Solo-Gabriele, H. M., Shibata, T., Plano, L. R. W., Elmir, S. M., Wanless, D., Bartkowiak, J., & Boiteau, R. (2010). Traditional and molecular analyses for fecal indicator bacteria in non-point source subtropical recreational marine waters. *Water Research, 44*(13), 3763–3772.

Sinton, L. W., Davies-Colley, R. J., & Bell, R. G. (1994). Inactivation of Enterococci and fecal coliforms from sewage and meatworks effluents in seawater chambers. *Applied and Environmental Microbiology, 60*(6), 2040–2048.

Su, Y., Sulavik, M., He, P., Makinen, K., Makinen, P. L., Fiedler, S., et al. (1991). Nucleotide sequence of the gelatinase gene (gelE) from *Enterococcus faecalis* subsp. liquefaciens. *Infection and Immunity, 59*(1), 415–420.

Tennstedt, T., Szczepanowski, R., Krahn, I., Pühler, A., & Schlüter, A. (2005). Sequence of the 68,869 bp IncP-1alpha plasmid pTB11 from a wastewater treatment plant reveals a highly conserved backbone, a Tn402-like integron and other transposable elements. *Plasmid 53*, 218–238.

Thanapalasingam, V. (2005). *Pollution status of the Sungai Skudai river system through heavy metals*. Master Thesis. Universiti Teknologi Malaysia, Johor, Skudai.

Trivedi, K., Cupakova, S., & Karpiskova, R. (2011). Virulence factors and antibiotic resistance in Enterococci isolated from food-stuffs. *Veterinární Medicína, 56*(7), 352–357.

Tsikrikonis, G., Maniatis, A. N., Labrou, M., Ntokou, E., Michail, G., Daponte, A., et al. (2012). Differences in biofilm formation and virulence factors between clinical and fecal enterococcal isolates of human and animal origin. *Microbial pathogenesis, 52*(6), 336–343.

Twikirize, J. M., & O'Brien, C. (2012). Why Ugandan rural households are opting to pay community health insurance rather than use the free healthcare services. *International Journal of Social Welfare, 21*(1), 66–78.

USEPA. (2012). Fecal bacteria: What are fecal bacteria and why are they important? Available at http://water.epa.gov/type/rsl/monitoring/vms511.cfm. Accessed 6 June 2012.

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., et al. (2004). Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in Enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. *Journal of Clinical Microbiology, 42*(10), 4473–4479.

Vankerckhoven, V., Huys, G., Vancanneyt, M., Snauwaert, C., Swings, J., Klare, I., et al. (2008). Genotypic diversity, antimicrobial resistance, and virulence factors of human isolates and probiotic cultures constituting two intraspecific groups of *Enterococcus faecium* isolates. *Applied and Environmental Microbiology, 74*(14), 4247–4255.

Vergis, E. N., Shankar, N., Chow, J. W., Hayden, M. K., Snydman, D. R., Zervos, M. J., et al. (2002). Association between the presence of enterococcal virulence factors gelatinase, hemolysin, and enterococcal surface protein and mortality among patients with bacteremia due to *Enterococcus faecalis*. *Clinical Infectious Diseases, 35*(5), 570–575.

Villar, C., de Cabo, L., Vaithiyanathan, P., & Bonetto, C. (1999). Pore water N and P concentration in a floodplain marsh of the Lower Paraná River*. *Hydrobiologia, 392*(1), 65–71.

Wade, T. J., Pai, N., Eisenberg, J. N. S., & Colford, J. M., Jr. (2003). Do US Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. *Environmental Health Perspectives, 111*(8), 1102.

Waters, C. M., Antiporta, M. H., Murray, B. E., & Dunny, G. M. (2003). Role of the *Enterococcus faecalis* GelE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. *Journal of Bacteriology, 185*(12), 3613–3623.

Wheeler Alm, E., Burke, J., & Spain, A. (2003). Fecal indicator bacteria are abundant in wet sand at freshwater beaches. *Water Research, 37*(16), 3978–3982.

Whitman, R. L., & Nevers, M. B. (2003). Foreshore sand as a source of *Escherichia coli* in nearshore water of a Lake Michigan beach. *Applied and Environmental Microbiology, 69*(9), 5555–5562.

Woodford, N., Morrison, D., Cookson, B., & George, R. (1993). Comparison of high-level gentamicin-resistant *Enterococcus faecium* isolates from different continents. *Antimicrobial Agents and Chemotherapy, 37*(4), 681–684.

Yoder, J. S., Blackburn, B. G., Craun, G. F., Hill, V., Levy, D. A., Chen, N., et al. (2004). Surveillance for waterborne-disease outbreaks associated with recreational water—United States, 2001–2002. *MMWR Surveillance Summaries, 53*(8), 1–22.

Zhang, X. X., Zhang, T., & Fang, H. H. P. (2009). Antibiotic resistance genes in water environment. *Applied Microbiology and Biotechnology, 82*(3), 397–414.