APPLICATION OF MULTILOCUS SEQUENCE ANALYSIS FOR MOLECULAR CHARACTERIZATION OF ENTEROCOCCI WITH VIRULENCE FACTORS RECOVERED FROM A TROPICAL RECREATIONAL BEACH

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Abstract. Partial gene sequences of phenylalanyl-tRNA synthase alpha subunit (*pheS*) and RNA polymerase alpha subunit (*rpoA*) were evaluated for species delineation and detection of recombination among enterococci populations recovered from a bathing beach impacted by low tide river flow. At inter-species level, a maximum similarity of 86.5% and 94.8% was observed among the enterococci *pheS* and *rpoA* sequence, respectively. A superimposed plot of delimited pair-wise similarity values obtained for 266 pair-wise observations revealed that while there was a harmony between species identity obtained from both genes, *pheS* was more discriminatory than *rpoA*. The difference was more pronounced for inter-species comparison. A number of putative recombination events between indigenous and non-indigenous strains was detected based on a library of aligned sequences. Virulence genes *cyl*, *esp*, *gelE* and *asa* were detected in 7, 22, 100 and 63%, respectively among river isolates but at lower proportion of 0, 20, 67 and 42%, respectively among beach water isolates. Random amplified polymorphic DNA profiling presented evidence suggesting low tide river as a source of fecal enterococci entering the recreation beach water. Multilocus sequence typing analysis of a number of *Enterococcus faecalis* isolates presented four sequence types, ST59, 117, 181 and 474. The presence of genetically diverse fecal enterococci with associated virulence traits and a background of recombination events in surface recreational water could present a potential public health risk.

Keywords: enterococci, MLST, *pheS*, RAPD, recombination, *rpoA*, sequence type

INTRODUCTION

The genus *Enterococcus* consists of gram-positive, catalase-negative, non-spore-forming, facultative anaerobic bacteria, which can occur both as a single coccus and in chains. Enterococci are important causes of hospital-acquired and community-acquired infection (Murray, 1990; Fisher and Phillips, 2009). Entero-

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cocci may possess a number of virulence factors, which are associated with the severity and duration of infections they cause. These include gelatinase, enterococcal surface protein, aggregation substance, cytolysin and hyaluronidase, encoded by *gelE*, *esp*, *asa*, *cyl*, and *hyl*, respectively (Jett *et al*, 1994; Hancock and Gilmore, 2002). Clinical isolates of enterococci show a lower diversity than those obtained from the environment with *Enterococcus faecalis* being the dominant species, arguably due to the virulence factors associated with this species (Fisher and Phillips, 2009). While the occurrence of virulence strains has been extensively studied among clinical enterococci (Padilla and Lobos, 2013), only recently did interest begin to emerge for the elucidation of virulence genes among enterococci recovered from recreational beach waters (Pinto *et al*, 2012; Santiago-Rodriguez *et al*, 2013). The dearth of published information partly explains why the ecology of virulence among environmental strains of enterococci is still not well understood (Santiago-Rodriguez *et al*, 2013).

Perhaps the most dreaded threat of enterococci is their ability to transfer and receive through exchange of plasmids or transposons, genetic material coding for antibiotic resistance or virulence in an inter- and intra-species manner (de Vera and Simmons, 1996; Kwon *et al*, 2012). This provides advantages to their survival under unusual environmental stresses and in part explains their increasing importance as nosocomial pathogens (Jett *et al*, 1994). Studies have suggested the possibility of this occurring in marine ecosystems (de Vera and Simmons, 1996; Davison, 1999; Rathnayake *et al*, 2011). Transmissibility of genetic factors through mating experiments has been demonstrated under laboratory conditions (Kwon *et al*, 2012), but there is a paucity of information on recombination events based on nucleotide sequence analysis of field samples. Given the sensitivity of a number or recombination detection algorithms, programs have been designed to predict potential recombination events from gene sequence libraries. Genetic recombination in bacteria where inter-species recombination is frequent, sequence exchange patterns can suggest subtle ecological and evolutionary links between certain species and barriers between others (Martin *et al*, 2010). Recombination is an important mechanism driving genetic variation and epidemic population structure, noticeable among particular species of the genus *Enterococcus* (Ruiz-Garbajosa *et al*, 2006).

The aim of the current study was to apply multilocus sequence typing (MLST) analysis with a specific reference to potential recombination events for the molecular characterization of potentially virulent enterococci recovered from a tropical recreational beach in the east coast of Malaysia.

MATERIALS AND METHODS

Study area

Teluk Chempedak is Kuantan's most popular beach, located 5 km east of Kuantan town center, Pahang. Chempedak River drains into the sea just overhead of the main bathing area. There is also a storm water drainage system, which empties into this river at the brink of the influx into the sea (Fig 1).

Sample collection

Thirty-six water samples were collected in triplicate using sterile glass bottles (1,000 ml) from the river draining into the sea at various locations, at the area of influx into seawater and at several locations on the bathing beach. Fifteen

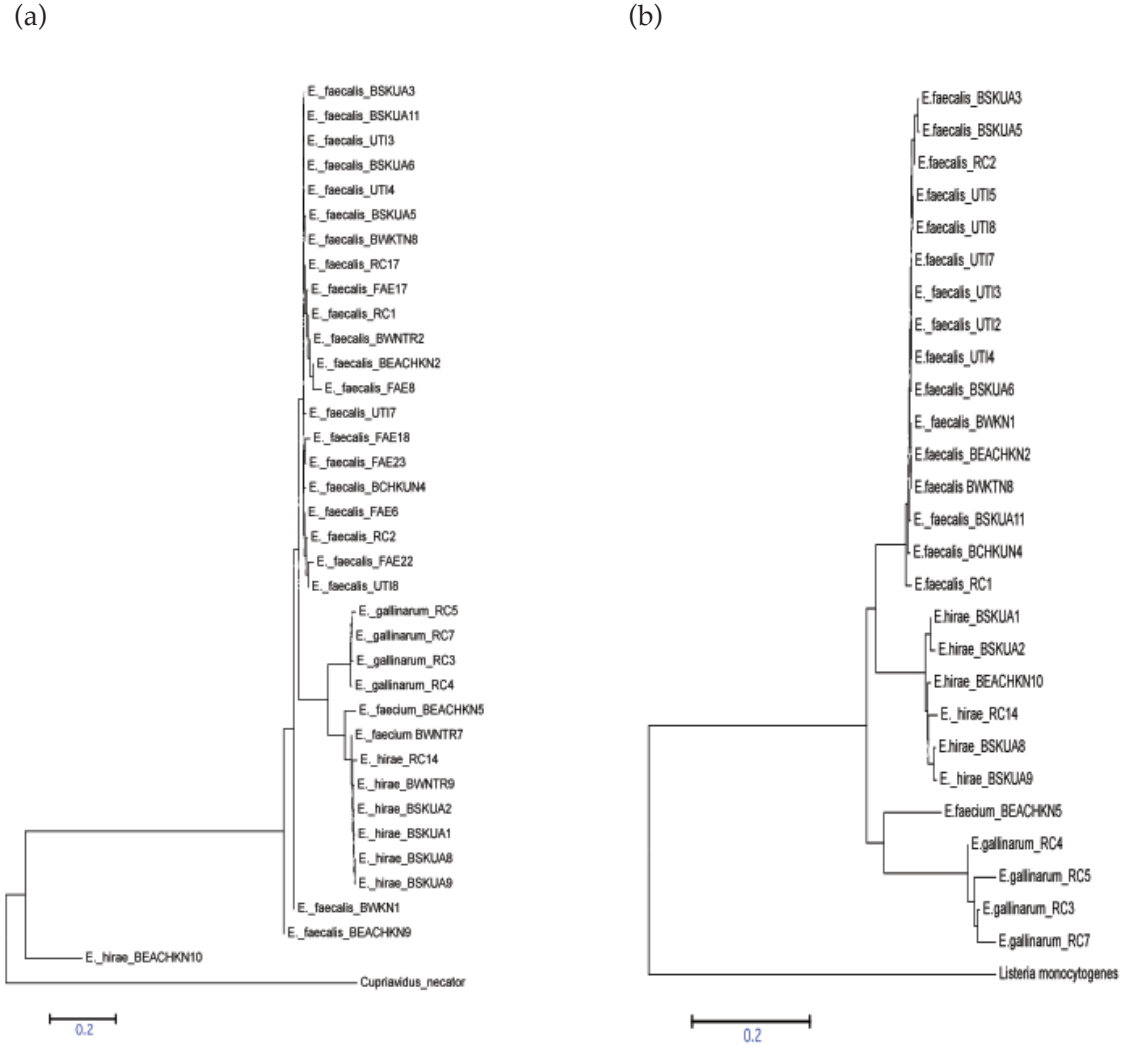

Fig 1–Neighbor-joining tree based on the *rpoA* gene sequences (n =36) and *pheS* gene sequences (n = 27) of enterococci s trains. Bootstrap percentages after 1,000 simulations are shown. *Cupriavidus nectator* and *Listeria monocytogenes* was included as outgroup for *rpoA* and *pheS,* respectively.

sand samples were also collected in sterile plastic containers. Fecal samples were collected at toilets proximate to the beach while catheters from urinary tract infection patients were also collected from a teaching hospital in Kuala Lumpur.

Isolation and enumeration of enterococci

Bacterial densities of enterococci from seawater samples were determined by membrane filtration method using Slanetz and Bartley(S+B) culture media (Oxoid, Hamshire, UK) incubated at 37°C for 24 to 48 hours. Characteristic colonies with red coloration were subjected to preliminary tests as described by Facklam and Elliot (1995) using tests for hydrolysis of bile esculin, catalase production, growth in NaCl (6.5%) and growth at 45.1°C.

Table 1
Primers used in the study.

Gene	Primer name	Sequence (5′– 3′)	Amplicon (bp)
Molecular characterization			
16S rRNA	*B27F*	AGA GTT TGATCC TGG CTC AG	1,300
	U1492R	GGT TAC CTT GTT ACG ACT T	
phenylalanyl-tRNA synthase (*pheS*) *PheS*	*pheS*-21-F	CAYCCNGCHCGYGAYATGC	600
	pheS-22-R	CCWARVCCRAARGCAAARCC	
RNA polymerase α subunit (*rpoA*)	*rpoA*-21-F	ATGATYGARTTTGAAAAACC	900
	rpoA-23-R	ACHGTRTTRATDCCDGCRCG	
RAPD genotyping			
Random primer	*D8635*	GAG CGG CCA AAG GGA GCA GAC	
Multi locus sequence typing			
glucose-6-phosphate dehydrogenase	*gdh-1*	GGCGCACTAAAAGATATGGT	530
	gdh-2	CCAAGATTGGGCAACTTCGTCCCA	
glyceraldehydes-3-phosphate dehydrogenase	*gyd-1*	CAAACTGCTTAGCTCCAATGGC	395
	gyd-2	CATTTCGTTGTCATACCAAGC	
phosphate ATP binding cassette transporter	*pstS-1*	CGGAACAGGACTTTCGC	583
	pstS-2	ATTTACATCACGTTCTACTTGC	
glucokinase	*gki-1*	GATTTTGTGGGAATTGGTATGG	438
	gki-2	ACCATTAAAGCAAAATGATCGC	
shikimate-5-dehydrogenase	*aroE-1*	TGGAAAACTTTACGGAGACAGC	459
	aroE-2	GTCCTGTCCATTGTTCAAAAGC	
xanthine phosphoribosyl-transferase	*xpt-1*	AAAATGATGGCCGTGTATTAGG	456
	xpt-2	AACGTCACCGTTCCTTCACTTA	
acetyl-CoA acetyltransferase	yiqL-1	CAGCTTAAGTCAAGTAAGTGCCG	436
	yiqL-2	GAATATCCCTTCTGCTTGTGCT	
Virulence markers			
Aggregation substance (asa+)	ASA 11	GCACGCTATTACGAACTATGA	375
	ASA 12	TAAGAAAGAACATCACCACGA	
Gelatinase (gel+)	GEL 11	TATGACAATGCTTTTTGGGAT	213
	GEL 12	AGATGCACCCGAAATAATATA	
Cytolysin (cyl+)	CYT I	ACTCGGGGATTGATAGGC	688
	CYT IIb	GCTGCTAAAGCTGCGCTT	
Enterococcal surface protein (esp+)	ESP 14F	AGATTTCATCTTTGATTCTTGG	510
	ESP 12R	AATTGATTCTTTAGCATCTGG	

Sequencing of *rpoA* and *pheS*

Sequence analysis of housekeeping *pheS* and *rpoA* was performed as described by Naser *et al* (2005). Primer pairs employed for PCR are listed in Table 1. PCR amplifications were performed in an Eppendorf Mastercycler (Hauppauge, NY) using an initial cycle of 94ºC for 3 minutes; 35 cycles of 94ºC for 1 minute, 46ºC for 1 minute (*rpoA*) or 47ºC for 1 minute (*pheS*), 72ºC for 1.5 minutes; and a final step of 72ºC for 10 minutes (Naser *et al*, 2005). PCR amplifications were performed in 0.2 ml reaction tubes

each with 25 µl of mixture composed of 0.1 µM each primer, 200 µM dNTPs, 1X PCR buffer, 2.5 mM $MgCl_2$ and 2U *Taq* polymerase. PCR amplicons were analyzed by 1% agarose gel-electrophoresis and directly sequenced in an ABI 3130XL 20 gene analyzer (Applied Biosystems, Foster City, CA).

Sequence analysis and construction of phylogenetic trees

The *pheS* and *rpoA* sequences were analyzed using NCBI BLAST search program and aligned with sequences of representative strains from the same phylogenetic group. All 16S rDNA sequences were checked for chimeras using Bellerophon software (Huber *et al*, 2004). The construction of phylogenetic trees was performed with Mega5 program using neighbor-joining method and Kimura's two-parameter model (Tamura *et al*, 2011). Reliability of the groups was evaluated by bootstrap analysis with 1,000 resamplings. Matrix global alignment tool (MatGat) (Campanella *et al*, 2003) was used to check for similarities among sequences using BLOSUM50 alignment matrix program. DNAsp was used to highlight polymorphisms in the library of sequences generated. Multiple alignments of *rpoA* and *pheS* sequences were employed to investigate putative recombination events. Statistical significance was set at $p = 0.05$. Each analysis was conducted four times to ensure repeatability of results. *E. faecalis* V583, *E. gallinarum* LMG12904, *E. hirae* ATCC 9790 were employed as reference strains.

Determination of virulence markers distribution in enterococci

Multiplex PCR assays were applied to identify virulence determinants, *asa*, *cylA*, *esp* and *gelE* as described by Vankerckhoven *et al* (2004). Details of primers and annealing temperatures are listed in Table 1. Reference strains were gratefully provided by Prof Shankar (University of Oklahoma, USA) and Dr Fatimah Lopez (Institute of Bioscience, Brazil). Primer pairs employed for PCR are listed in Table 1. PCR amplifications were performed in an Eppendorf Mastercycler (Hauppauge, NY) using 0.2 ml reaction tubes each with 25 µl of mixture composed of 0.1 µM each primer (*asa* and *gel*) or 0.2 µM each primer (*cyl* and *esp*), 200 µM dNTPs, 1X PCR buffer, 2.5 mM $MgCl_2$ and 2 U *Taq* polymerase. Primer pairs employed for PCR are listed in Table 1 and the thermocycling (Eppendorf Mastercycler, Huappauge, NY). PCR conditions were as follows: an initial cycle of 94°C for 3 minutes; 35 cycles of 94°C for 1 minute, 56°C for 1 minute, 72°C for 1.5 minutes; and a final step of 72°C for 10 minutes. PCR amplicons were separated by agarose gel-electrophoresis, directly sequenced as described above and compared with known sequences by BLAST program (http://www.ncbi.nlm.nih.gov).

Genetic diversity determination of enterococci by random amplified polymorphic DNA (RAPD)-PCR typing

In order to subtype *E. faecalis* recovered during the study, a total of 35 isolates were subjected to RAPD-PCR analysis as described by Barbosa *et al* (2009). D8635 primer was used (First Base Sdn Bhd, Seri Kembangal, Malaysia) (Table 1) and *E. faecalis* MMH 594 was the reference strain. Band patterns obtained by a UV transilluminator were processed using ImageJ (NIH, Bethesda, MA). Pearson's coefficient and agglomerative clustering via unweighted pairs group matching algorithm were employed for statistical analysis, and subsequently the RAPD-PCR profiles were analyzed using PyElPh version 2.6.5 (Python Software Foundation®, Wilminaton, DE).

MLST

A number of isolates carrying virulence genes were selected for MLST using a total of seven housekeeping genes (Table 1). PCR conditions were as described by Ruiz-Garbjosa *et al* (2006). PCR amplifications were performed in an Eppendorf Mastercycler (Hauppauge, NY) using 0.2 ml reaction tubes each with 25 µl of mixture composed of 0.1 µM each primer, 200 µM dNTPs, 1X PCR buffer, 2.5 mM $MgCl_2$ and 2U *Taq* polymerase. Primer pairs employed for PCR are listed in Table 1. PCR thermocycling conditions were as follows: an initial cycle of 94ºC for 3 minutes; 35 cycles of 94ºC for 1 minute, 52ºC for 1 minute, 72ºC for 1.5 minutes; and a final step of 72ºC for 10 minutes. PCR amplicons were purified by agarose gel-electrophoresis and sequenced as described above. For each locus, a distinct allele number was assigned to every different sequence, in accordance with the *E. faecalis* MLST database (http://efaecalis.mlst.net/).

Nucleotide sequence accession numbers

Nucleotide sequences determined in this study were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The 16S rDNA sequences were submitted under GenBank accession no. KC890838-KC890842 andKC707577-707586, *pheS* sequences under accession no. KC707575, KC890824-KC890842, and KC963174-KC963180, and *rpoA* sequences under no. KC707576 and KC963138-KC963173.

RESULTS

Species diversity of enterococci

A total of 96 enterococci isolates were recovered from the beach water environment and the low tide river water flowing into the beach (Table 2). There was a marked difference in the diversity of enterococci species recovered from beach sand (BS) and beach water (BW). Among BW isolates, *E. casseliflavus* and *E. faecalis* was detected at the highest percentage (47% and 42%, respectively), while the least occurring species was *E. hirae* and *E. faecium* (both 5%). On the other hand, in BS, *E. hirae* was the most predominant (65%), followed by *E. faecalis* (27%). Notably a high proportion of *E. gallinarum* (45%) was detected in Chempedak River, while it was not detectable in BW and BS samples. *E. hirae* (27%) and *E. faecalis* (23%) were also present among enterococci recovered from Chempedak River water samples. Analysis of 2x2 contingency tables of *E. faecalis* and *E. faecium* frequencies in BW and BS showed no association in the occurrence of these two species. Chi-square analysis of the diversity data revealed significant difference ($X^2 = 70.86$, df = 16, $p < 0.0001$) in the frequency of occurrence of enterococci diversity from the various sites sampled during the study.

Heterogeneity of *rpoA* sequences among enterococci samples

The partial *rpoA* sequences (n=36) had a G+C content of 38.31 ± 0.99%, similar to the value obtained by Naser *et al* (2005) and consistent with the average G+C content for the total genome of enterococci (Klein, 2003; Paulsen *et al*, 2003). The strains in our study were clearly delineated as they produced distinct branches (Fig 1a). Considering inter-species similarities, a maximum of 94.8% similarity for *rpoA* was observed in the study, and intra-species similarity was at a maximum of 99.8%. As for *E. hirae* strains, intra-species similarity was at a maximum of 97.6% and a minimum of 85.6%. *E. hirae* BEACHKN10 had a notably low inter- and intra-species similarity, with minimum and maximum value

of 30.3% and 52.2%, respectively. Apart from *E. faecalis* BWKN1, BEACHKN9 and BWNTR7, which had notably low inter- and intra-species similarity values (30.3-52.2%), intra-species similarity was generally high with a maximum of 99.8% and most strains clustered together in the *rpoA*-based phylogeny. Intra-species similarity was at a maximum of 98.2% and a minimum of 85.1% for *E. gallinarum* strains. Based on *rpoA* sequence analysis, the closest neighbors of *E. hirae* were *E. faecium* (94.8% *rpoA* similarity), *E. gallinarum* (87.7%) and *E. faecalis* (84.7%). Also based on *rpoA* sequence similarity, the closest neighbors of *E. faecalis* were *E. gallinarum* (84.7%) and *E. faecium* (83.9%). The *rpoA* phylogenetic tree revealed two sub-clusters of *E. faecum* and *E. hirae* within the *E. faecium* species group. Within the *E. faecium* species group, *E. faecium* BEACHKN5 isolated from BW samples was highly related to *E. hirae* BSKUA8 and BSKUA9, with 94.4% *rpoA* similarity. A maximum similarity of 98.2% of *rpoA* sequence was observed for intra-species comparison among *E. gallinarum* strains, which were notably associated only with Chempedak River water samples and clustered together in the phylogenetic tree.

Heterogeneity of *pheS* sequences among enterococci samples

The partial *pheS* sequences (n = 27) in our study had a G+C content of 41.5 ± 2.5%, similar to values obtained by Naser *et al* (2005) and consistent with the average G+C content for the total genome of enterococci (Klein, 2003; Paulsen *et al*, 2003). Analysis of the *pheS* sequences revealed a higher degree of resolution compared with *rpoA* for enterococci species differentiation (Fig 1b). At the inter-species level, a maximum similarity of 86.5% was observed among the enterococci *pheS* sequences. For *E. hirae* strains, intra-species similarity was at a maximum of 91.8% and a minimum of 52.2%, and they were well differentiated in the species group forming a distinct cluster, unlike in the *rpoA* tree where they clustered with *E. faecium* BEACHKN5. For *E. faecalis*, intra-species similarity was generally high at a maximum of 99.8% and most strains were clustered together as was observed in the *rpoA*-based phylogeny. Based on *pheS* sequence analysis, the closest neighbors of *E. hirae* were *E. faecalis* (86.5% *pheS* similarity), *E. faecium* (81.5%) and *E. gallinarum* (79.3%). Furthermore, based on *pheS* sequence similarity, the closest neighbors of *E. faecalis* were *E. faecium* (90.4%) and *E. gallinarum* (81.2%). The generally lower similarity values obtained for *PheS* sequences comparisons at the inter- and intra-species level indicate the relatively higher efficiency of differentiation based on *pheS* sequence analysis, consistent with the findings of Naser *et al* (2005).

Comparison between *rpoA* and *pheS* sequences

Going by the harmony in terms of species identity correctness, analysis of the results showed that both *rpoA* and *pheS* sequences are useful for the classification of the populations of enterococci used in this study. MatGat aligned *rpoA* (n = 36) and *pheS* (n = 27) sequences were analysed for similarity by pair-wise comparisons using the BLOSUM50 scoring matrix. Analysis of a superimposed plot of pair-wise similarity values obtained for a total of 266 pair-wise observations for enterococcal *pheS* and *rpoA* sequences showed similarity in peaks and trend lines for both *rpoA* and *pheS* sequences, revealing that while there was a harmony between species identity obtained from both genes, *pheS* was more discriminatory than *rpoA* (Fig 2). The difference was more

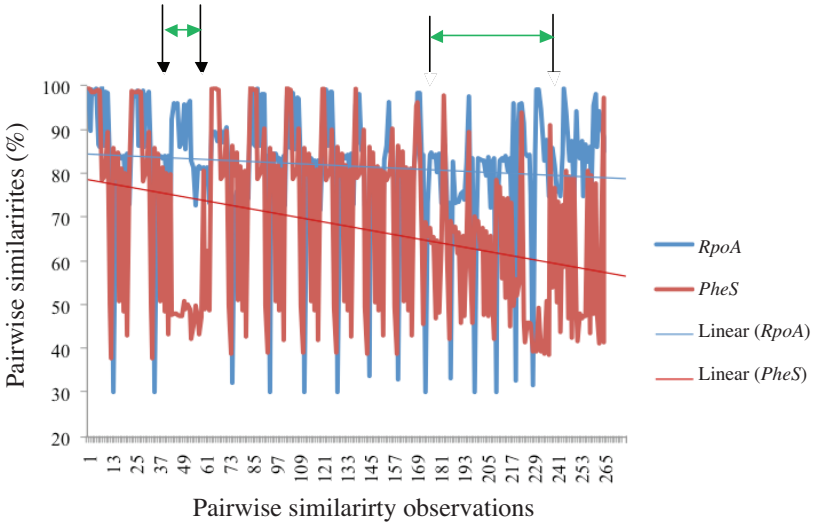

Fig 2–Pair-wise similarity based on MatGat aligned *rpoA* and *pheS* sequences using BLOSUM50 scoring matrix. The inter-species differences in *rpoA* and *pheS* sequences are marked with green arrows.

pronounced for inter-species comparison (marked with green arrows).

Putative recombination analysis

A library of *rpoA* and *pheS* sequences obtained in this study were tested for putative recombination events using a recombination detection program that combines a number of approaches, *viz* BOOTSCAN, GENECONV, Maximum Chi-Square (MAXCHI), CHIMAERA, sister scanning method (SISCAN), and 3SEQ method (Martin *et al*, 2010). Recombinant analysis thus presents information on possible recombination breakpoints, names of sequences in the dataset that are closely related to the presumed parents of the recombinant sequence, the approximated probability values of observing a recombination signal, the number of sequences in the dataset with similar signals detected by different recombination detection methods, and a graph showing evidence used by the program to infer which of the sequences used to detect the recombination (Fig 3a-c).

Sequences of *E. faecalis* strains isolated from urinary tract infections (UTI) from hospital patients were included in the recombination analysis to simulate a scenario where non-invasive clinical strains find their way into recreational water possibly via infected individuals who utilize the beach water. Similarly *E. faecalis* recovered from fecal samples at toilets proximate to the beach were included to simulate a scenario of direct fecal contamination. Previous studies have reported the occurrence of several hidden pipes that discharge sewage directly to the seawater in the vicinity of the study area (Hamzah *et al*, 2011). Results obtained from the recombinant analysis of *rpoA* sequences showed that there were a number of possible recombination events based on the library of aligned sequences (designated in pink regions in Fig 3a-c). Seven sequences showed evidence of the same recombination event. In the putative recombinant *E. hirae* BS1 (Fig 3a-c), a start break point at 146 (position 169 in alignment) and end breakpoint at 776 (position 836 in alignment) was observed. Similar recombination events were observed among *rpoA* sequences of *E. hirae* BS2, BS8, BS9, and RC14; *E. faecium* BEACHKN5 and *E. gallinarum* RC3, RC4 and RC5. In these putative recombinant strains, analysis indicated that the major

Table 2
Frequency of the distribution of *Enterococcus* species diversity among the five sampled sites.

Species	Number of isolates (%) from each isolation site					*Enterococcus* spp distribution	*p*-value
	1	2	3	4	5		
E. casseliflavus	9 (9)	2 (2)	2 (2)	1 (1)	0	14 (14)	
E. hirae	1 (1)	1 (1)	18 (19)	6 (6)	5 (5.21)	31 (32)	
E. gallinarum	0	0	0	10 (10)	8 (8.33)	18 (19)	0.0001
E. faecium	1 (1)	0	0	0	0	1 (1)	
E. faecalis	8 (8)	7 (7)	6 (6)	5 (5)	6 (6.25)	32 (33)	
Total enterococci per site	19 (20)	10 (10)	26 (27)	22 (23)	19 (19.79)	96 (100)	

Isolation site: 1, beach water (bathing area); 2, point where Chempedak River empties into recreational beach water; 3, beach soil; 4, Chempedak River water; 5, Chempedak River soil. A *p*-value <0.05 was considered significant.

parent was *E. faecalis* BCHKUN4 and the minor parent *E. faecalis* FAE8, suggesting that these BW isolates and enterococci from fecal sources are possible sources for the putative recombinants obtained in our study.

Occurrence of virulence markers among enterococci samples

Virulence gene *cyl*, *esp*, *gelE* and *asa* was detected in 7, 22, 100 and 63%, respectively among river isolates but at lower proportion of 0, 20, 67 and 42%, respectively among BW isolates (Table 3). That *cyl*-positive isolates were detected only among river area and not among BW isolates implicated urban flow as potential source of *cyl*-carrying enterococci influx into recreational bathing water. All fecal isolates possessed *esp* and 58% of BW isolates also possessed *esp*, which was absent among all BS isolates. Apart from the fecal and BW isolates, only the Chempedak River isolates (23%) carried *esp* gene.

RAPD-PCR and MLST analysis

All *E. faecalis* isolates recovered were subjected to RAPD analysis. A total of 16 RAPD unique profiles were obtained from a selected library of 35 *E. faecalis* isolates reflecting a diverse variability among the strains (Fig 4). The RAPD profiles presented three main clusters, with *E. faecalis* BWNTR strains forming a sub-cluster in the main cluster of *E. faecalis* strains recovered from Chempedak River. Also, most of the fecal isolates of *E. faecalis* formed a sub-cluster along with strains from Chempedak River. These results confirmed the findings of the recombinant detection analysis.

Based on a combination of their harborage of virulence genes and RAPD profiles, four *E. faecalis* isolates were selected for MLST analysis (Table 4). Strain RC19 was observed to be ST18, which shares 6/7 tested loci with ST6 known to be a member of the clonal complex 2 (CC2), a widely distributed multi-drug resistant strain that has been previously associated with hospital outbreaks (Novais *et al*, 2004; Freitas *et al*, 2009, 2011a). It was of interest to note that this isolate was recovered from the river at low tide, which drains

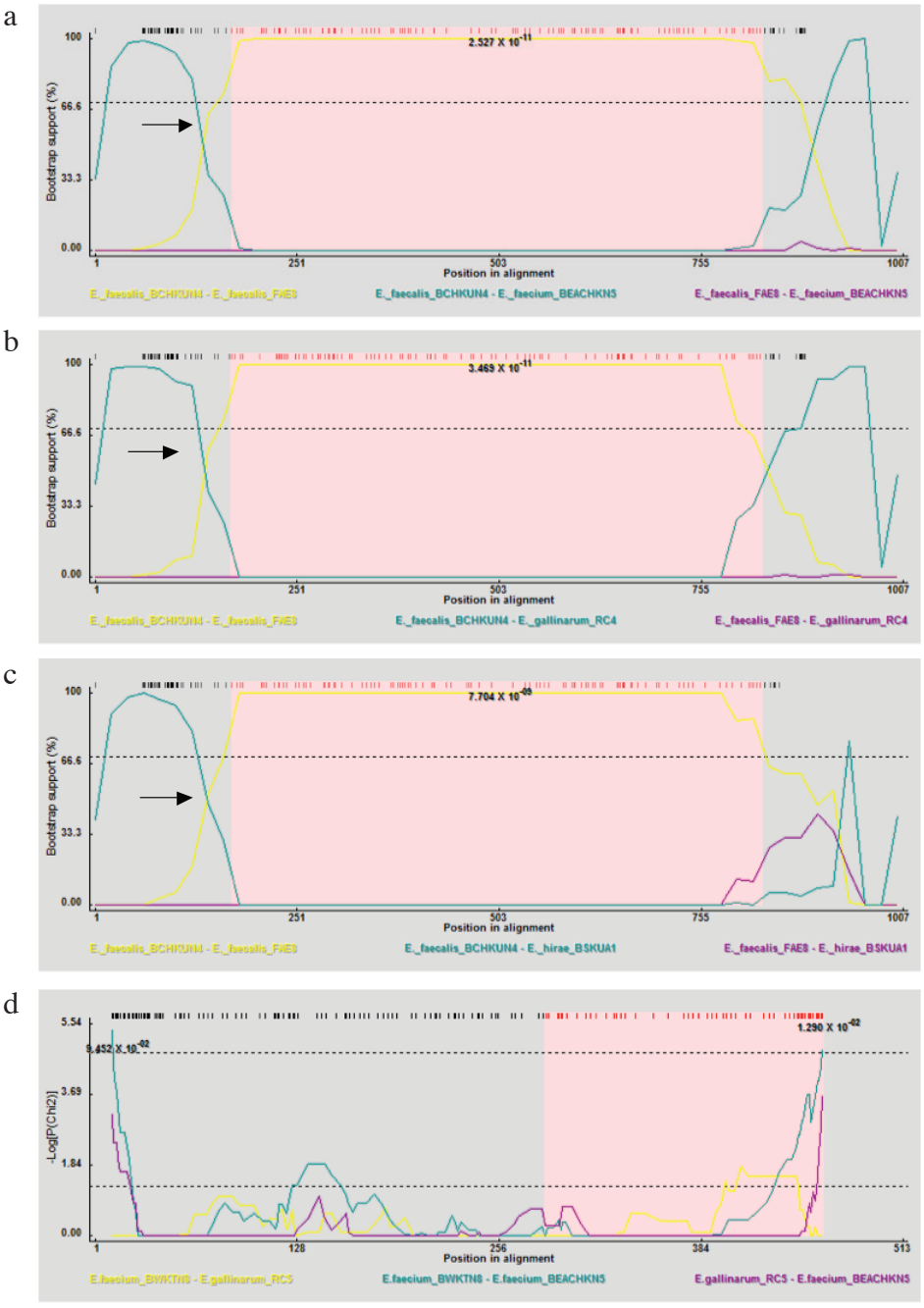

Fig 3–BOOTSCAN and MAXCHI plots showing the locations of possible recombination events associated with multiple aligned RpoA sequences. In (a-c), the Y-axis indicates percent bootstrap values that support the clustering of each recombinant sequence with the parental strains. The area outlined in pink demarcates the potential recombination regions. Boot strap values over 70% are taken as significant. High degrees of bootstrap support between two different sequence pairs are indicative of potential recombination events. Crossover sites are indicated by arrows. Potential breakpoints are displayed as the left and right boundaries of the pink region.

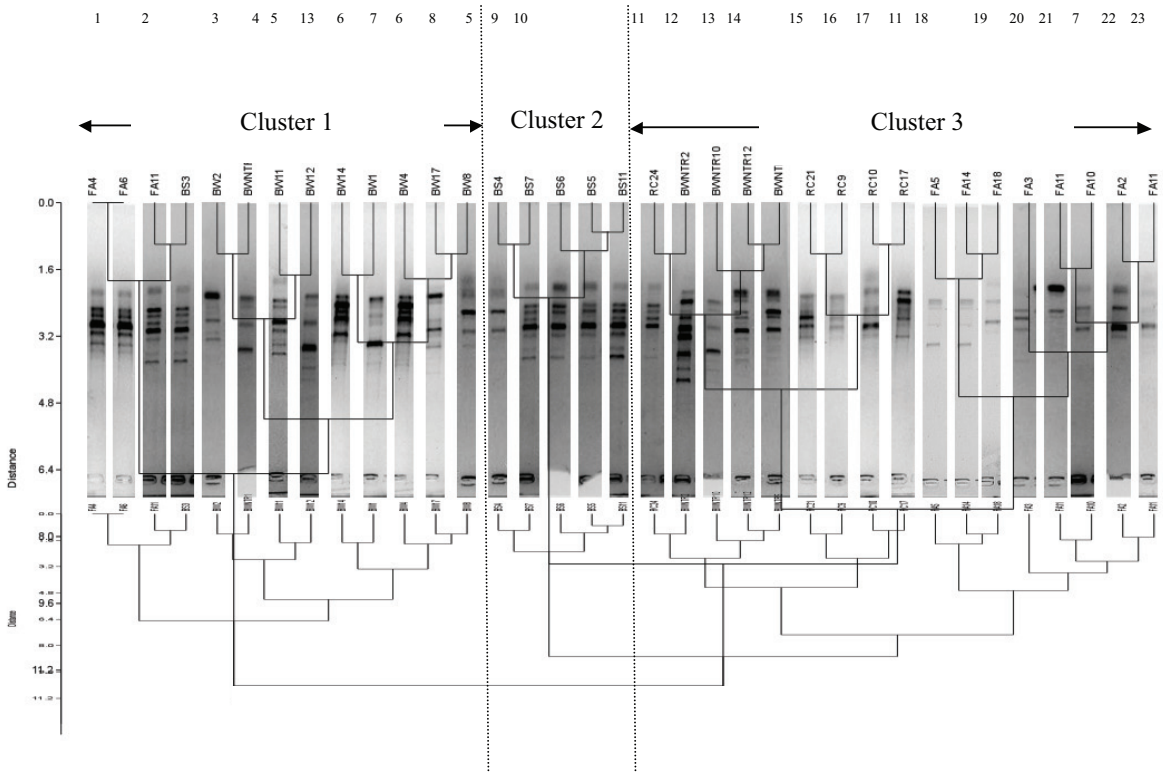

Fig 4–*D8635* primer-based RAPD profiles of the 35 *E. faecalis* strains tested in the study for the purpose of detecting the source of fecal enterococci in the considered recreational beach water. Pearson's coefficient and agglomerative clustering via unweighted pairs group matching algorithm were employed to generate the profiles.

into the seawater used for recreational purposes. Analysis of the MLST data revealed another isolate (RC59) as a ST59 strain, which was recovered also from the low tide river. The fourth strain selected (BW17) was recovered from recreational bathing water and was found to be ST117. It also harbored *esp*, *asa* and *gel* genes.

DISCUSSION

In this study, enterococci species recovered from a recreational beach and an urban-flow influenced river were genotypically identified. The results obtained showed that the diversity of enterococci species recovered was significantly associated with the sampling sites. Agreeably, the marked variance in the heterogeneity of enterococci species that were recovered in the various sites may reflect the influx of non-indigenous enterococci species. Our observation corroborates the view of Plan (2010) on the influx into coastal and marine ecosystems.

In the analysis of *rpoA* and *pheS* sequences obtained in this study, two critical answers were sought, namely, without additional operational costs associated with cloning to obtain complete gene sequences, could partial *rpoA* and *pheS* gene sequences suffice for identification and, more importantly, for typing of an enterococci library comprised largely of

Table 3
Prevalence of single/multiple virulence markers with species diversity and isolation site.

S/no	Virulence marker	Beach area ($n = 55$) Proportion (%)					River area ($n = 41$) Proportion (%)					Total enterococci no. (%)
		Ec	Eh	Eg	Efm	Efs	Ec	Eh	Eg	Efm	Efs	
1	*cyl*$^+$	0	0	-	0	0	0	9	11	-	0	3 (3)
2	*esp*$^+$	46	5	-	0	19	0	27	28	-	9	20 (21)
3	*asa*$^+$	69	20	-	0	48	0	73	61	-	64	49 (51)
4	*gelE*$^+$	92	40	-	100	76	100	100	100	-	100	78 (81)
5	*asa*$^+$*gelE*$^+$	69	20	-	0	48	0	73	61	-	64	49 (51)
6	*esp*$^+$*gel*$^+$	46	5	-	0	19	0	27	28	-	9	20 (21)
7	*esp*$^+$*asa*$^+$*gel*$^+$	46	5	-	0	19	0	27	28	-	9	20 (21)
8	*cyl*$^+$*esp*$^+$*asa*$^+$*gel*$^+$	0	0	-	0	0	0	9	11	-	0	3 (3)

Ec, *Enterococcus casseflavus;* Eh, *Enterococcus hirae;* Eg, *Enterococcus gallinarum;* Efs, *Enterococcus faecalis*; Efm, *Enterococcus faecium*; *cyl*+, strains carrying gene coding for cytolysin; *esp*+, strains carrying gene coding for enterococcal surface protein; *asa*+, strains carrying gene coding for aggregation substance; *gelE*+, strains carrying gene coding for gelatinase.

Table 4
Characteristics of the four *E. faecalis* isolates selected for MLST analysis.

Strain	gdh	gyd	pstS	gki	aroE	xpt	yqil	Sequence type (ST)	Colonal complex (CC)	Virulence marker
E. faecalis RC9	14	2	18	10	16	2	12	59	N/A	*asa*+, *gelE*+
E. faecalis BW15	14	1	18	57	16	35	12	474	N/A	*asa*+, *gelE*+
E. faecalis BW17	1	1	9	6	1	1	1	117	CC21	*esp*+, *asa*+, *gelE*+
E. faecalis RC19	12	5	3	7	6	1	5	181	CC2	*esp*+, *asa*+, *cyl*+, *gelE*+

cyl+, strains carrying gene coding for cytolysin; *esp*+, strains carrying gene coding for enterococcal surface protein; *asa*+, strains carrying gene coding for aggregation substance; *gelE*+, strains carrying gene coding for gelatinase; N/A, singleton with no available clonal complex.

environmental strains. Ideally for a gene sequencing-based method to be termed as robust for typing purposes, it should satisfactorily facilitate the clustering into similar groups the strains collected from similar sources. In line with previous studies (Svec *et al*, 2005; Sukontasing *et al*, 2007; Tanasupawat *et al*, 2008), our study proved the applicability of these genes as being robust for identification of environmental enterococci. However, the clustering observed from the dendograms revealed that typing could not be achieved based on a partial gene sequence analysis of these two genes. This corroborates the findings of Naser *et al* (2005).

Rapid classification schemes using MLSA should involve a universal set of protein coding genes that are widely distributed as single copies among bacterial genomes, at levels below saturation for the analyzed groups and importantly, that they are not unusually prone to recombination (Naser *et al*, 2005). However, a limitation of that study was the source of the enterococci library tested, which was more or less restricted to mainly clinical settings and environmental enterococci isolates were not considered. Meanwhile, because of the vulnerability of the environment to anthropogenic pollution, environment-derived strains are faced with a variety of selection pressures that may allow for recombination events.

While potential recombination events and recombinants were identifiable among the populations of beach and river water isolates, there was no evidence of recombinants events among all UTI strains examined. The lack of evidence for recombination events among clinical strains in our study is in agreement with the findings of Naser *et al* (2005). However, it should be noted that the clinical strains tested were restricted to non-invasive enterococci associated with UTI. The possibility may exist of possible recombination events within the hospital environments leading to the emergence of rare clones (Kawalec *et al*, 2007; Pinto *et al*, 2012). In our study, putative recombination events detected were restricted to environmental and fecal isolates, suggesting possible linkages between these factors. A number of possible explanations exist for the observations. The fact that the major parent of the putative recombinant is *E. faecalis* BEACHKN4 (BW isolate) and minor parent *E. faecalis* Fae8 (fecal isolate) (Fig 3a) may suggest the intrusion of fecal enterococci from some fecal polluted source or via direct sewage discharge into the recreational beach water. A soak-away failure at the study location and possibility of hidden drains discharging sewage into seawater have previously been reported (Tiong, 2001; Hamzah *et al*, 2011). It could be possible that these strains are thus able to exchange genetic materials with indigenous BW enterococci strains, which were thereafter detected during the recombination analysis. These putative recombinants ultimately accumulate in recreational water environment and share genetic materials with other BW strains as suggested by the recombination events detected between beach isolates BCHKUN4 and BEACHKN5 (Fig 3a).

A common way that bacteria adapt to different environmental conditions is via the acquisition of alleles from a different species of bacteria that are subsequently integrated into the original allele (Schmidt and Hensel, 2004; Bennett, 2008; Guinane *et al*, 2010). Our study also found evidence of recombination events between strains from urban river emptying into the recreational beach water as in the case of *E. faecalis* BCHKUN4 and *E. gallinarum* RC4 recombination (Fig 3b). This transfer

of genetic material apparently was not restricted at the intra-species level as evidence of recombination was also observed between *E. faecium* Beachkn5 and *E. faecalis* BEACHKUN4 (Fig 3a), between *E. faecalis* BEACHKUN4 and *E. gallinarum* RC4 (Fig 3b) and between *E. faecalis* BEACHKUN4 and *E. hirae* BSKUA1 (Fig 3c).

The results obtained in our recombination analysis of *rpoA* sequence was in concert with the test for positive selection using Nei Gojobori method calculated using a 1,000 replicate bootstrapping method. The Maximum Likelihood method adopted for the molecular clock test for the obtained topology with and without the molecular clock constraints in Tamura-Nei model (Tamura *et al*, 2011) also confirmed that the rate of evolution among the sequences analyzed was not equal (InL-with clock: -797.238, InL-without clock: -747.136, $p = 0.00672$). The results obtained was also in conformity with equality of evolution test using Taijaima's relative approach ($x^2 = 5.93$, $p = 0.01489$). The null hypothesis of equal rates between lineages based on *rpoA* sequences was thus rejected based in the observed *p*-values.

Unlike the *rpoA* sequences that demonstrated several recombination events, only a single putative recombinant was detected among the *pheS* sequences examined. In this putative recombinant strain, it was observed that the major parent was *E. faecalis* BW8 and the minor parent *E. gallinarum* RC5, suggesting that these enterococci from beach water and Chempedak River are possible sources of the putative recombinant *E. faecium* BEACHKN5. Presented in Fig 3d is the Maxchi plot of potential recombination events among the *pheS* sequences, with the left and right boundaries of the pink region indicating breakpoint positions. A limitation, however, with this approach is that MAXCHI provides information on the positions of potential breakpoints but does not give information regarding the extent of recombinant regions. The potential recombination event based on MAXCHI however was considered a false positive because it was observed to be incongruent with other detection methods (*eg*, BOOTSCAN or Sawyers test-based GENECONV method). Thus it appears that, based on the library of *pheS* sequence analyzed, this gene may be more stable to environmental selection pressure for recombination than *rpoA*.

The results obtained in our recombination analysis of *pheS* sequence were in agreement with other complementary tests conducted on the *pheS* sequences. The Maximum Likelihood method adopted for the molecular clock test for the obtained topology with and without the molecular clock constraints in Tamura-Nei model (Tamura *et al* 2011) confirmed the rates of evolution among the examined sequences to be equal (InL-with clock: -840.903, InL-without clock: -829.973, $p = 0.9998$). The results obtained was also in conformity with equality of evolution test using Taijaima's relative approach ($x^2 = 2.13$, $p = 0.14413$). The null hypothesis of equal rates between lineages based on *pheS* sequences was thus accepted. This observation also corroborates the analysis from the recombination analysis that suggest the river emptying into the beach water along with other fecal sources as being responsible for the preponderance of *esp*-carrying enterococci in beach water. In developing nations, urban rivers often serve as sewers for communities who simply discharge waste into rivers. Some of these flowing waters may be rich in fecal contamination and may account for the *esp*-containing enterococci strains

that eventually reach the sea.

In the current study, RAPD-PCR was used to characterize 35 selected strains of *E faecalis* producing a total of 23 RAPD unique profiles. This result indicated a high variability of enterococci sub-species diversity among the *E. faecalis* strains. The high variability observed is in concert with the findings of another study (Son *et al*, 1999) where 19 RAPD-types were reported from a total of 19 tested *E. faecum* isolates. In a microbial source tracking attempt by Martin *et al* (2009), over ninety RAPD-types were reported albeit based on a library of 596 enterococci isolates. RAPD is an inexpensive, efficient, and sensitive alternative typing method for recognizing genetic differences between closely related bacteria. However, its application has a number of limitations. Such problems with reproducibility and discriminatory power, frequently cited in the literature, are surmountable by precise optimization procedure allowing the achievement of reliable conditions for each species analyzed (Gzyl and Augustynowkz, 1998). Apart from initial species specific evaluation of the RAPD working conditions conducted in this study, two main primers (M13 and D8635) were screened and one retained based on the achievement of stable and informative amplification patterns for the purpose of discrimination among the tested *E. faecalis* strains. Analysis of the RAPD profiles presented three main clusters (Fig 4). Notably again, *E. faecalis* BWNTR strains formed a sub-cluster in the main cluster of *E. faecalis* strains recovered from Chempedak river (RC). Also, most of the fecal isolates of *E. faecalis* formed a subcluster along with strains from river Chempedak. These results thus present evidence to suggest the source of these *E. faecalis* strains detected in the considered bathing beach water may be due to fecal contaminated urban river flows that drain into the recreational water. A number of studies have also adopted RAPD-PCR as an important tool to indicate patterns of niche-specific associations of enterococci strains and to provide evidence that enterococci sub-species associate with specific environment (Son *et al*, 1999; Anderson, 2005; Rathnayake *et al*, 2011).

Our observation of the recovery of CC2 strains in non-hospital settings, however, corroborate a recent report of ST strains belonging to this clonal complex from liquid manure and sewage (Freitas *et al*, 2009). The occurrence of ST59 has been previously reported among chickens, fecal sample from a healthy pig and from hospitalized patients (Ruiz-Garbajosa *et al*, 2006; Kawalec *et al*, 2007). Although previously characterized CC21 strains usually harbor less antibiotic resistance and fewer virulence traits than isolates of other CCs (Ruiz-Garbajosa *et al*, 2006; Kawalec *et al*, 2007; McBide *et al*, 2007), the possibility of recombination in beach water settings could herald successful acquisition of diverse genetic elements that might facilitate their persistence and spread in environments under selective antibiotic pressure. A previous study (Freitas *et al*, 2009) reported the isolation of members of this clonal complex from both hospital patients and from pig farms. In other studies, CC21 isolates have been frequently observed in the global *E. faecalis* collection among isolates of diverse origins, including hospitalized patients, non-hospitalized individuals, meat and farm animals (Ruiz-Garbajosa *et al*, 2006, 2007). This seems to highlight the dispersion of this CC strain in different ecological settings.

Among STs reported in this study, ST117 and ST181 were only previously

reported as novel STs in a study conducted in Cuba (Quinones *et al*, 2009) and another study on waterways in Australia (Rahnayake *et al*, 2011). The regional geographical proximity of the latter to Malaysia may be a useful premise of a ST181 clonal regional spread. Nevertheless, the local availability of virulence genes is a crucial determinant of the extent to which these factors are acquired by epidemic clones through horizontal gene transfer (Novais *et al*, 2004; Nilsson *et al*, 2009). It is difficult, however, to generalize in the case of the occurrence in beach environment settings in Malaysia of the novel ST117, originally detected in Cuba.

Only three studies have previously reported the genetic variability of enterococci in Malaysia, all of which focused on clinical and animal strains. Weng *et al* (2012) reported the occurrence of ST18 and ST596 strains among isolates recovered from hospital patients. In a later study Weng *et al* (2013) reported ST17, ST78, ST203 and ST601 strains from clinical specimens. In the most recent study, Getachew *et al* (2013) reported ST4, ST6, ST87, ST108, ST274 and ST244 from a total of 11 isolates recovered from humans, chickens and pigs. Our report is the first of the genetic variability of enterococci strains recovered from beach water environment in Malaysia using MLST.

Considering the growing evidences that clinical resistance is intimately associated with environmental bacteria (de Vera and Simmons, 1996; Prabhu *et al*, 2007; Abriouel *et al*, 2008), it is increasingly obvious that research activities need to be expanded to include environmental microorganisms. The observation in the current study of one of the strains recovered from a river that drains into beach water as being a member of a hospital-adapted clonal complex (CC2) is an issue of concern. A recent study also reported the occurrence of hospital-adapted *E. faecium* CC17 from a beach environment (Pinto *et al*, 2012). It could be that both the low tide river, from which this strain was isolated, along with the storm water drainage, which empties into it, are acting as reservoirs for this ST and other potentially virulent strains. Subsequent rainfall events could dislodge them and ultimately they are dispersed into recreational beach water.

A limitation in this study, however, relates to financial restrictions that limit the number of strains analyzed by MLST, making it difficult to generalize on an overall representation of the *E. faecalis* population just based on the prevalence of a few clonal complexes. It is not feasible to identify a possible clonal expansion of a particular strain in the study location.

In conclusion, the findings of our study emphasizes the need for more studies on the characterization of enterococci strains from beaches, particularly those recovered from those receiving significant influx from polluting rivers and storm drainage systems. Arguably, there is also the need for inclusion of these environments in the global epidemiological research agenda.

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