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Occurrence of *Enterococcus* species with virulence markers in an urban flow-influenced tropical recreational beach

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ABSTRACT

Median enterococci counts of beach water samples gradually increased at statistically significant levels ($\chi 2$: 26.53, df: 4; $p < 0.0001$) with increasing proximity to river influx. The difference in proportion of antibiotic resistant enterococci in beach water and river water samples was statistically significant ($p < 0.05$) for the tested antibiotics with river isolates generally presenting higher resistance frequencies. Virulence genes *cyl*, *esp*, *gelE* and *asa* were detected at varying frequencies (7.32%, 21.95%, 100% and 63.41% respectively) among river isolates. On the other hand, the prevalence of these genes was lower (0%, 20%, 67.27% and 41.82% respectively) among beach water isolates. Multi-Locus-Sequence-Typing analysis of *Enterococcus faecalis* presented four sequence types (ST) one of which shared six out of seven tested loci with ST6, a member of the clonal complex of multi-drug resistant strains associated with hospital outbreaks.

1. Introduction

The impact of external influence on surface water quality is well documented (Bhaduri et al., 2001; Schippmann et al., 2013; Mallin et al., 2000). Anthropogenic influences (urbanization, industrial and agricultural activities) as well as complex natural processes (changes in precipitation inputs, erosion, and weathering of crustal materials) are all implicated in the degradation of surface water quality (Zhang et al., 2009). These characteristically impair their use for recreation or other purposes (Peng et al., 2005).

Surface water contamination in tropical and subtropical environments has been associated with waterborne diseases (Peng et al., 2005). Specifically, rivers in Malaysia have been reportedly treated as open sewers with attendant waste discharges which ultimately reach the sea (DailyExpress: Rubbish Ending Up in the sea, 2013). Pollutants from municipally-influenced sources may carry diverse bacteria which could directly pose threats to the health of recreational beach users (Barrell et al., 2000; Hamilton et al., 2010). Enterococci are commonly used as indicators of faecal contamination in recreational waters (Barrell et al., 2000). Studies that highlight enterococci diversity may play critical epidemiological roles in source pollution monitoring of surface waters. To date, there is no published study on the genetic variability of enterococci recovered from recreational beach water in Malaysia.

Enterococcus spp. are documented to be intrinsically resistant to a number of antibiotics including cephalosporins, penicillinase-resistant penicillins, and clinically available concentrations of lincosamides and aminoglycosides (Fisher and Phillips, 2009). Apart from the notoriety of members of this genus for antibiotic resistance, enterococci may possess a number of virulence factors which are associated with the severity and duration of infections caused by them. These include gelatinase, enterococcal surface protein (*Esp*), aggregation substance (*asa*), cytolysin (*cyl*) and hyaluronidase (*hyl*) Jett et al., 1994; Vergis et al., 2002; Semedo et al., 2003. While the occurrence of virulence strains has been extensively studied among clinical enterococci (Padilla and Lobos, 2013), studies are only recently beginning to emerge on the elucidation of virulence genes among enterococci recoverable from recreational beach waters is (Brownell et al., 2007; Santiago-Rodriguez et al., 2013; Layton et al., 2009; Rathnayake et al., 2012). The scanty published information partly explains why the ecology of antibiotic resistance among environmental strains of enterococci is still not well understood (Santiago-Rodriguez et al., 2013; Rathnayake et al., 2012). Furthermore, there is a dearth of information on the molecular

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determination of virulence markers among enterococci recoverable from tropical recreational beach waters (Santiago-Rodriguez et al., 2013).

The aim of the current study is, thus, to determine via molecular characterization, the species prevalence, diversity, antimicrobial resistance and virulence markers among enterococci recovered from a tropical recreational beach in the East Coast of Malaysia.

2. Materials and methods

2.1. Study area description

The coast of northern Pahang, Malaysia is composed of the three prominent bays (Cendur, Cerating and Beserah) and the minor bays of Pelindung and Chempedak (Tiong, 2001). Teluk Chempedak is Kuantan's most popular beach located 5 km east from Kuantan town centre, Pahang, Malaysia. Teluk Chempedak is usually crowded during the weekends with people engaging in a number of sporting activities. Along the Chempedak bay, River Chempedak drains into the sea just overhead the main bathing area at Teluk Chempedak. There is also a stormwater drainage system that empties into this river at the brink of influx into the sea (Fig. 1).

2.2. Sample collection

Bathing water samples for both beaches were taken from areas close to the swash zone of the bathing beach at locations that cover the length of the beach (Fig. 1). Water samples were collected also from the river draining into the sea at various locations and at the area of influx into the seawater. Sterile glass bottles (1000 ml) were used to collect water samples in triplicates. Sand samples were collected in sterile plastic containers. Faecal samples were collected at toilets proximate to the beach.

2.3. Isolation and enumeration of enterococci

Bacterial densities of enterococci from seawater samples were determined by membrane filtration method (A.P.H.A, 1999), using Slanetz and Bartley (S + B) culture media. Plates were incubated at 37 °C for 24–48 h. Preliminary tests on presumptive enterococci were performed as previously described (Dada et al., 2013). All isolates were designated as members of the genus *Enterococcus* by the PCR detection of the *tuf* gene as described by Creti et al. (2004). Isolates were also confirmed by the amplification of *sodA* and *ddl* genes (*Enterococcus faecalis* and *Enterococcus faecium*) (Jackson et al., 2004; Dutka-Malen et al., 1995) and 16SrRNA sequencing (for identification of other *Enterococcus* spp.). Primers are listed in Table 1.

2.4. Multi Locus Sequence Typing

Multi-drug resistant *E. faecalis* isolates with virulence traits were selected for Multi Locus Sequence Typing (MLST). MLST analysis involved a total of seven housekeeping genes: *gdh* (glucose-6-phosphate dehydrogenase), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *pstS* (phosphate ATP binding cassette transporter), *gki* (putative glucokinase), *aroE* (shikimate 5-dehydrogenase), *xpt* (shikimate 5-dehydrogenase), and *yiqL* (acetyl-coenzyme A acetyltransferase) (Table 1) (Ruiz-Garbajosa et al., 2006). PCR conditions for all amplification reactions were as follows: initial denaturation at 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min; and final extension at 72 °C for 7 min. Reactions were performed in 25-μl volumes using *Taq* polymerase (FirstBase Sdn Bhd). The resulting PCR amplicons were purified with a kit (Qiagen) and sequenced using an ABI 3130XL 20 genetic analyzer (Applied Biosystems). For each locus, a distinct allele number was assigned to the obtained nucleotide sequences in accordance with the *E. faecalis* MLST database (http://efaecalis.mlst.net/).

2.5. Nucleotide sequence accession numbers

Nucleotide sequences determined in this study were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The deposited information was simultaneously made available to EMBL in Europe and the DNA Data Bank of Japan. *sodA* gene sequences were submitted under GenBank accession numbers KC603859-KC603862; *ddl* gene sequences were submitted under GenBank accession numbers KC594678-KC594682; 16S rRNA gene sequences were submitted under GenBank accession numbers KC890838-KC890842, KC707577-707586.

2.6. Detection of antibiotics resistance and resistance determinants among Enterococci

Antibiotic resistance among enterococci isolated from the beaches was determined by the single disc Kirby-Bauer diffusion method using Mueller–Hinton Agar (Oxoid, UK) NCCLS: Performance Standards for Antimicrobial Disk Susceptibility Testing, 2003. A total of 96 enterococci isolates recovered from Teluk Chempedak beach water and beach sand, River Chempedak water and sand were tested for resistance to seven (7) antibiotic groups. Antibiotics tested include vancomycin (V) (glycopeptides) (30 μg), kanamycin (K) (30 μg), streptomycin (S) (25 μg) (aminoglycoside), ampicillin (A) (10 μg) (B-lactam), tetracycline (T) (30 μg) (tetracycline), chloramphenicol (C) (30 μg) (chloramphenicol) and nitrofuranoin (N) (50 μg) (nitrofuran) (Oxoid, UK). Diameters of zones of inhibition were recorded in mm and interpreted as sensitive or resistant using breakpoints for enterococci as proposed by the National Committee for Clinical Laboratory Standards (NCCLS: Performance Standards for Antimicrobial Disk Susceptibility Testing, 2003).

2.7. Determination of virulence-markers distribution in enterococci

Polymerase chain reaction (PCR) assays were applied to amplify virulence determinants {aggregation substance (*asa*), cytolysin (*cylA*), enterococcal surface protein (*esp*), gelatinase (*gelE*)} (Vankerckhoven et al., 2004). Details of primers are listed in Table 2. For each primer, initial optimization experiments were conducted to ascertain optimal pcr conditions for $MgCl_2$ and annealing temperatures. Multiplex PCR conditions used in this study included an initial activation step at 95 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a single cycle of 7 min at 72 °C. Reference strains were gratefully provided by Prof. Shankar of the Department of Medicinal Chemistry and Pharmaceutics, University of Oklahoma and Dr. Fatimah Lopez, Institute of Bioscience, Brazil. PCR amplicons of *asa, cylA, esp, gelE* genes were confirmed by DNA sequencing with an ABI 3130XL 20 genetic analyzer (Applied Biosystems). The DNA sequences were blasted for sequence similarity with annotated sequences at http://www.ncbi.nlm.nih.gov.

2.8. Phenotypic assays

All isolates were subjected to gelatinase assay as described by Cariolato et al. (2008) using Brain–Heart Infusion agar plates supplemented with 10 g/L peptone and 30 g/L gelatine. Assay for proteolytic activity was conducted by detecting casein hydrolysis in MHA containing 3% (w/v) skimmed milk. Biofilm formation was determined by crystal violet assay in polystyrene microtiter