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# Genetic diversity of *Enterococcus faecalis* isolated from environmental, animal and clinical sources in Malaysia

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ABSTRACT

*Enterococcus faecalis* ranks as one of the leading causes of nosocomial infections. A strong epidemiological link has been reported between *E. faecalis* inhabiting animals and environmental sources. This study investigates the genetic diversity, antibiotic resistance and virulence determinants in *E. faecalis* from three sources in Malaysia. A total of 250 *E. faecalis* isolates were obtained consisting of 120 isolates from farm animals, 100 isolates from water sources and 30 isolates from hospitalized patients. Pulse-field gel electrophoresis-typing yielded 63 pulsotypes, with high diversity observed in all sources (D = ≥0.901). No pulsotype was common to all the three sources. Each patient room had its own unique PFGE pattern which persisted after six months. Minimum inhibitory concentrations of Vancomycin, Gentamicin, Penicillin, Tetracycline, Nitrofurantoin, Levofloxacin, Ciprofloxacin and Fosfomycin were evaluated. Resistance to Tetracycline was most prevalent in isolates from farm animals (62%) and water sources (49%). Water isolates (86%) had a higher prevalence of the *asa1* gene, which encodes for aggregation substance, whereas clinical (78%) and farm animal isolates (87%) had a higher prevalence of the *esp* gene, encoding a surface exposed protein. This study generates knowledge on the genetic diversity of *E. faecalis* with antibiotic resistance and virulence characteristics from various sources in Malaysia.

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## Introduction

*Enterococcus faecalis* is found in a variety of environments, such as soil, water, plants, and animals [1]. In humans, as well as in other mammals, these microbes are mainly found in the gastrointestinal tract as commensals. However, *E. faecalis* may become an opportunistic pathogen in individuals whose immune systems are compromised [2]. The virulence associated genes in human pathogenic *E. faecalis* encode among others a collagen-binding protein (*ace*) [3], an aggregation substance (*asa1*) [4], a haemolysin activator (*cylA*) [4], an endocarditis antigen (*efaA*) [5], a surface protein (*esp*) [4], gelatinase (*gelE*) [6] and two recently identified putative surface antigens, *EF0591* and *EF3314* [7]. *E. faecalis* has also been shown to acquire resistance to a wide range of antibiotics [8]. As a result, enterococci have emerged as one of the leading therapeutic challenges associated with enterococcal infections including urinary tract infections (UTI) [2]. Around the world *E. faecalis* remains one of the most frequently recovered species from enterococcal infections in humans [9].

Due to the prevalence of *E. faecalis* in nosocomial infections, studies have suggested hospital settings as a source for antibiotic-resistant strains [10]. Additional studies suggest environmental sources including animals and water can serve as important sources for antibiotic resistant *E. faecalis* strains [1] as human populations, animal populations, and the environment are all interconnected [1]. Selection and persistence of antibiotic resistance might be attributed to a variety of factors including horizontal transfer of resistance genes among bacteria, the misuse or overuse of antibiotics in humans and animals, and environmental contamination through livestock slurry and plant wastewater. The rate of development of resistance appears to have accelerated in the past decade and today multiple antibiotic resistant bacteria constitute a global problem [11].

It is important to investigate the genetic relationships between microbes, such as *E. faecalis*, that are found in both the environment and hospitals, as a possible relationship between the different sources may be established. Although a number of studies have investigated the prevalence and characteristics of antibiotic resistance among enterococci in clinical and environmental settings in

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Malaysia [12–17], such studies are typically limited to vancomycin-resistant enterococci and/or studies of a limited geographical area. In this study, *E. faecalis* from the feces of farm animals, water sources and hospital patients in Malaysia were characterized. The genetic relationships, virulence determinants and antibiotic susceptibilities shared between human and environmental *E. faecalis* isolates from different sources were assessed. In addition, these same characteristics were assessed from the same location after a period of six months to assess the persistence of each type of isolate in each source.

## Materials and methods

### *Study site and sample collection*

Sampling was carried out in two states representing different geographical regions in Malaysia; Selangor (West Malaysia) and Sabah (East Malaysia). Study sites comprised of chicken and cattle farms, wastewater treatment plants, rivers and hospitals. All farms and water sources were located within a 15 km radius of the hospitals in Selangor and Sabah respectively. The sampling areas in Sabah comprised of small to medium residential communities surrounded by rural agricultural regions as opposed to Selangor which included sampling areas around semi-urban development constituting smallholder farms. Sampling was conducted at two different sampling times, June and December 2014. Details of the sampling procedure and the distribution of samples obtained in this study can be found in Supplementary material 1.

### *Isolation and identification of* E. faecalis

Suspected *E. faecalis* appearing as typical black to brown colonies on BAA agar, indicating esculin hydrolysis, were transferred on Slanetz and Bartley (SlaBa) agar (Oxoid, UK) and identified by growth and biochemical reactions as described by Olutiola et al. [18].

### *Confirmation of* E. faecalis *identity by sequencing of 16S ribosomal DNA*

All presumptive *E. faecalis* isolates, including the clinical *E. faecalis* isolates obtained from hospital patients, were further characterized by 16S rDNA sequencing as a confirmation from phenotypic testing as proposed by Marchesi et al. [19]. Total DNA was extracted using the GF-1 Bacterial DNA Extraction Kit (Vivantis, Malaysia). Primers were obtained from First BASE Laboratories, Malaysia. Species identification was determined from the best-scoring reference sequence of the BLAST output and whether the best-scoring reference sequence in the database had a sequence identity of 98% with e-values $10^{-5}$ and at least 96% query coverage.

### *PFGE analysis*

Pulse Field Gel Electrophoresis (PFGE) was performed (3 replicates per isolate) subsequent to DNA digestion with *Sma*I (Promega, USA) as described by Weng et al. [17]. The PFGE marker (Promega, USA) containing lambda concatemers and lambda-digested *Hind*III fragments was used as a size standard. Comparison of the PFGE fingerprints was analyzed with Cliqs 1D Pro software (Cliqs 1D Pro, USA).

### *Antibiotic susceptibility testing*

The minimum inhibitory concentration (MIC) was determined for all *E. faecalis* isolates against a range of antibiotics using the broth microdilution technique according to standard recommendations [20]. The list of antibiotics tested in this study is provided in Supplementary material 2. These antibiotics were chosen because they are either used in both human medicine and animal husbandry or because previous studies have reported *E. faecalis* resistance to them [21]. All antibiotics were purchased from Oxoid (UK) and Nacalai Tesque (Japan). The results were interpreted according to the cut-off levels proposed by CLSI guidelines [20].

### *Screening for* vanA *and* vanB *genes*

All isolates were subjected to PCR for *vanA* and *vanB* genes according to Dutka-Malen et al. [22]. Primers were obtained from First BASE Laboratories, Malaysia.

### *Putative virulence markers*

All primers for testing the presence of putative virulence markers were selected according to Creti et al. [7]. Primers for all virulence markers tested in this study are listed in Supplementary material 3. Primers were obtained from First BASE Laboratories, Malaysia.

### *Statistical analysis*

The prevalence of antibiotic resistance phenotype to each antibiotic among *E. faecalis* isolates from all sources was compared using the chi-squared test. A P-value of $<0.05$ was considered to be statistically significant. Simpson's index of diversity (D) was calculated [23] to assess the differentiation of *E. faecalis* pulsotypes by PFGE. PFGE analysis was based on Dice similarity coefficient and unweighted pair group method using arithmetic averages (UPGMA) clustering with position tolerance and optimization coefficient of 1.5%.

## Results

### *Sample collection*

In this study, one isolate per sample was haphazardly picked for analysis. A total of 250 *E. faecalis* isolates were obtained throughout this study; 120 from farm animal feces, 100 from water sources and 30 from hospital patients (Supplementary material 1).

### *Diversity of* Enterococcus faecalis *isolates by PFGE*

The analysis based on the dendrogram generated from the PFGE profiles grouped the *E. faecalis* isolates into 63 pulsotypes (with $\geq$90% similarity) with 44 clonal populations and 19 isolates that were treated as unique. The PFGE patterns of samples from Selangor and Sabah showed distinct differences. The complete dendrogram is shown in Fig. 1.

A total of 27 pulsotypes for isolates from farm animal feces, 47 for isolates from water sources and 8 for clinical isolates were obtained. Isolates from the same farm clustered together, with the exception of four isolates in pulsotypes XLII and XLVIII which displayed identical PFGE patterns between Farm A and Farm B, as shown in Fig. 1. There was no overlapping of PFGE patterns between isolates from chicken and cattle feces. All isolates from animal drinking water showed similar PFGE patterns to those from farm animals with respect to the farms sampled. Isolates from river water and wastewater showed large genetic variability. *E. faecalis* from wastewater did not cluster according to the two wastewater treatment plants that were sampled, although farm samples did cluster according to the source farm. In addition, this study found identical PFGE patterns between two pulsotypes consisting both

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