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Research paper

Comparative genome analysis of multiple vancomycin-resistant *Enterococcus faecium* isolated from two fatal cases

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ABSTRACT

Enterococcus faecium is both a commensal of the human intestinal tract and an opportunistic pathogen. The increasing incidence of enterococcal infections is mainly due to the ability of this organism to develop resistance to multiple antibiotics, including vancomycin. The aim of this study was to perform comparative genome analyses on four vancomycin-resistant *Enterococcus faecium* (VRE_{fm}) strains isolated from two fatal cases in a tertiary hospital in Malaysia. Two sequence types, ST80 and ST203, were identified which belong to the clinically important clonal complex (CC) 17. This is the first report on the emergence of ST80 strains in Malaysia. Three of the studied strains (VREr5, VREr6, VREr7) were each isolated from different body sites of a single patient (patient Y) and had different PFGE patterns. While VREr6 and VREr7 were phenotypically and genotypically similar, the initial isolate, VREr5, was found to be more similar to VRE2 isolated from another patient (patient X), in terms of the genome contents, sequence types and phylogenomic relationship. Both the clinical records and genome sequence data suggested that patient Y was infected by multiple strains from different clones and the strain that infected patient Y could have derived from the same clone from patient X. These multidrug resistant strains harbored a number of virulence genes such as the *epa* locus and pilus-associated genes which could enhance their persistence. Apart from that, a homolog of *E. faecalis bee* locus was identified in VREr5 which might be involved in biofilm formation. Overall, our comparative genomic analyses had provided insight into the genetic relatedness, as well as the virulence potential, of the four clinical strains.

1. Introduction

Enterococci are commensals in the gastrointestinal tracts of humans and animals but some members of this genus are also opportunistic nosocomial pathogens which can cause diseases associated with bloodstream and urinary tract (Willems and van Schaik, 2009). Treatment of enterococcal infections is challenging due to the intrinsic and acquired resistance of enterococci to multiple antibiotics, including the last-line drugs such as vancomycin and daptomycin (Hollenbeck and Rice, 2012).

The first reported cases of vancomycin-resistant enterococci (VRE) occurred in the 1980s in the United Kingdom and France (Leclercq et al., 1988; Uttley et al., 1988). Since then, VRE are increasingly reported worldwide, including United States, Europe, and Asia (Bonten et al., 2001; Kuo et al., 2014). Resistance to vancomycin is typically mediated by one of the nine *van* gene clusters (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, *vanN*). Among them, *vanA* and *vanB* are the predominant resistance genotypes observed (Hollenbeck and Rice, 2012). Horizontal transfer of these genes to other pathogens has been a big concern. In fact, the conjugative transfer of enterococcal *vanA* gene to *Staphylococcus aureus* strain has been reported (Zhu et al., 2013). This interspecies transfer of resistance can result in highly resistant pathogens which are difficult to treat with the currently available antibiotics.

A rapid increase in the *Enterococcus faecium* infections has been observed worldwide since late 1980s, coinciding with the acquired vancomycin resistance (Treitman et al., 2005). In the United States, 87% of *E. faecium* recovered from nosocomial infections are vancomycin-resistant (Edelsberg et al., 2014). Molecular and comparative genomic studies showed that hospital-associated (HA) *E. faecium* strains are different from community-associated (CA) strains where mobile genetic elements and antimicrobial resistance genes are enriched in the HA strains (Qin et al., 2012).

Most of the HA strains belong to the clonal complex 17 (CC17) based on multilocus sequence typing (MLST) (Top et al., 2008). Strains from the CC17 pose specific traits that enable them to persist in the clinical environment. These include resistance to ampicillin and quinolone, and a pathogenicity island which carries the *esp* gene encoding enterococcal surface protein (Esp) putatively involved in biofilm formation and endocarditis (Heikens et al., 2012; Top et al., 2008). Other genes that contribute to virulence in *E. faecium* include *acm* which encodes for collagen binding adhesin that contributes to endocarditis (Nallapareddy et al., 2008) and ebp_{fm} operon which encodes for pili that are associated with biofilm formation and urinary tract infection (Nallapareddy et al., 2011).

The occurrence of VRE has been reported in several East Asian countries such as China, Taiwan, Japan, and Korea (Cha et al., 2012;

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Kuo et al., 2014; Matsushima et al., 2012; Zheng et al., 2007). In Malaysia, the first confirmed case of HA VRE was reported in 2006 in Hospital Kuala Lumpur (HKL) in a patient with chronic renal failure (Zubaidah et al., 2006). Other local studies reported a low prevalence of VRE (1–2%) with *E. faecium* being the most common species isolated (Ibrahim et al., 2010, 2011). Despite its low prevalence, the growing problems raised by VRE, such as reduced therapeutic options against infections caused by this pathogen, are a major public health concern in Malaysia. Hence, a better understanding on the pathogenicity, resistance, and persistence of this organism is important for VRE infection controls.

In this study, we applied pulsed field gel electrophoresis (PFGE) and whole genome sequencing (WGS) to four clinical vancomycin-resistant *Enterococcus faecium* (VRE_{fm}) strains isolated from two fatal cases in a tertiary hospital in Kuala Lumpur, Malaysia. We aimed to elucidate the genetic relatedness of these strains through comparative genomic analysis and determine the virulence factors and antimicrobial resistance determinants harbored by these strains. Our analyses had identified multiple antibiotic resistance genes and virulence gene in the four genomes. Most importantly, our results revealed infection of VRE_{fm} strains from different clones in a single patient, as well as possible spread of the pathogen between the two studied cases.

2. Material and methods

2.1. Clinical data collection and ethical approval

Clinical data including treatments and microbiological results were retrieved from the patient information database and clinical records kept in the hospital. This study had obtained ethical approval from the University of Malaya Research Ethics Committee (UMREC) with ethical approval number 20159–1661.

2.2. Bacterial strains

Four VRE_{fm} strains, VRE2, VREr5, VREr6, and VREr7 were from the culture collection of the Biomedical Science Laboratory, University of Malaya. These strains were previously isolated from patients admitted to University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia between January and June 2011. VRE2 was isolated from the blood of patient X and was the first VRE_{fm} isolated during that period (an index case as based on retrospective hospital records). VREr5, VREr6, and VREr7 were isolated from the cerebrospinal fluid (CSF), blood, and urine of a patient warded in the Intensive Care Unit (ICU), patient Y, respectively. These three strains were isolated at one-week interval, with VREr5 being the first isolate, followed by VREr6 and VREr7.

2.3. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed as previously described (Turabelidze et al., 2000), with slight modifications. Briefly, the bacteria were first lysed in a combination of lysozyme (100 mg/ml) and mutanolysin (10 kU/ml) at 37 °C for 4 h. Chromosomal DNA was then prepared in agarose gel block and digested with restriction enzyme *Sma*I (Promega, Madison, WI, USA) at 25 °C. The restriction fragments were separated by PFGE in 0.5 × TBE buffer for 20 h at 14 °C in a CHEF Mapper system (Bio-Rad, CA, USA) using pulsed times of 3.5–25 s and 1–5 s. *Xba*I-digested *Salmonella enterica* ser. Braenderup H9812 was used as the DNA size marker. The PFGE fingerprints were analyzed using BioNumerics version 6.0 software (Applied Maths, Kortrijk, Belgium). The quantitative differences among the banding patterns were defined by the Dice coefficient. Cluster analysis was determined based on the unweighted pair group method with averages (UPGMA), using a position tolerance 1.5%.

2.4. Antimicrobial susceptibility test

The antibiograms of the four sequenced strains were determined using Kirby-Bauer disc diffusion method (CLSI, 2016). For vancomycin, gentamicin and teicoplanin, minimum inhibitory concentrations (MICs) were determined using E-test strips (BioMérieux, Marcy-l'Étoile, France). The Clinical and Laboratory Standard Institute (CLSI) guidelines were used to interpret the results (CLSI, 2016).

2.5. Biofilm assay

To study the biofilm forming ability of the studied strains, crystal violet assay was performed using the protocol described by Baldassarri et al. (2001) with slight modifications. Biofilm was allowed to grow at 37 °C for 48 h and the optical density (OD) of eluted crystal violet stain was measured at 590 nm wavelength. The true OD readings of each strain were acquired after deducting the negative control, which contained only the growth medium. The biofilm forming ability of the studied strains was scored as previously described (Chelvam et al., 2014; Stepanović et al., 2000). The experiment was repeated three times to ensure reproducibility.

2.6. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) was performed to confirm the biofilm forming ability of the studied strains. Biofilm was grown in an eight-well chamber slide at 37 °C for 48 h. Visualization of biofilm was performed as previously described (Jurcisek et al., 2011). Confocal images were collected using a Leica TCS SP5 microscope. The images were viewed by using the LAS AF Lite software (Leica). The image stacks were acquired using ImageJ software (Fiji, ImageJ, Wayne Rasband National Institutes of Health).

2.7. DNA sequencing, assembly, and annotation

Whole genome sequencing of the four VRE_{fm} strains was performed on the Illumina Miseq platform, version 2.0 with reads coverage ranged from 78 × to 108 ×. Genome assembly was carried out using CLC Genomic Workbench version 5.1 (CLC Bio, Aarhus, Denmark) and the annotation was done using RAST (Rapid Annotation using Subsystem Technology), Prodigal and Blast2GO (Aziz et al., 2008; Conesa et al., 2005; Hyatt et al., 2010) as previously described (Yap et al., 2014).

2.8. In silico multilocus sequence typing

Multilocus sequence typing (MLST) was performed as described by Homan et al. (2002). The genome sequences were uploaded to the PubMLST website (http://pubmlst.org/efaecium/) in which the seven housekeeping genes (*atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, *adk*) were compared against the database to predict the respective allele numbers. Sequence types (STs) were defined based on the allelic profiles of the seven loci in the database.

2.9. Phylogenomic analysis of global strains of E. faecium

For phylogenomic analysis, 16 representative draft genomes of clinical *E. faecium* from ten different countries (Austria, United States of America, Netherlands, France, China, Africa, Portugal,

United Kingdom, Israel, Denmark) with varied sequence types (ST203, 17, 78, 117, 18, 210, 27, 26, 414, 160, 64) were included in our phylogenomic analysis. The names and respective Genbank accession numbers of the sixteen strains are as follows: Aus0085 (CP006620.1), Aus0004 (CP003351.1), TX16 (CP003683.1), E1133 (AHWR00000000.1), E155 (AUWX00000000.1), E1904 (AHXQ00000000.1), E0120 (AHWI00000000.1), E2560 (AHYI00000000.1), E1185 (AHWS00000000.1), E161 (JXZA00000000.1), LCT-EF128 (AJUP00000000.1), E1731 (AHXO00000000.1), E6045 (AHYL00000000.1), E1392 (AHWV01000046), E0333 (AHWL00000000.1), VRE84 (AIVF00000000.1). Genome sequences were submitted to the Reference Sequence Alignment-based Phylogenic Builder (RealPhy) (Bertels et al., 2014) for the identification of sites that are relevant for the phylogenomic study. *E. faecium* Aus0085 was chosen as the reference genome as this strain shares similar features with our Malaysian strains and has the same sequence type (ST203) as two of these strains. Aus0085 is a clinical VRE isolate with a complete genome and classically used for comparative genomics (Lam et al., 2013). The generated multiple genome sequence alignments were used to construct an approximately-maximum-likelihood tree using FastTreeMP (Price et al., 2010) as previously described (Yap et al., 2014).

2.10. Comparative genome analysis

The genome sequences of the four analyzed strains were aligned with the reference strain, *E. faecium* Aus0085, using Mauve 2.3.1. (Darling et al., 2004) and comparative analysis was performed. The homologous regions between the compared strains and their respective strain-specific regions were obtained by extracting the orthologous file from the Mauve alignment. The nucleotide and amino acid sequences of the predicted homologous regions were aligned and validated through BLASTn and BLASTp. A circular genomic map of the comparison was constructed using BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011). Insertion sequence (ISs) elements were analyzed by IS Finder (Siguier et al., 2006). Prophage loci were identified using PHAST (Zhou et al., 2011). The presence of CRISPR was identified using CRISPRfinder (Grissa et al., 2008).

2.11. Identification of putative virulence genes and antibiotic resistance determinants

Putative virulence-associated genes were identified by comparing the amino acid sequences of the four studied strains against the sequences of enterococcal virulence factors found in the Virulence Factors of Pathogenic Bacteria Database (VFDB) through BLASTn (Chen et al., 2005). The results were validated through BLAST against the non-redundant database. To identify antibiotic resistance genes, the nucleotide sequences were submitted to the Antibiotic Resistance Gene Database (ARDB) (Liu and Pop, 2009) to generate respective resistance profiles with corresponding resistance genes. The nucleotide sequences were also submitted to ResFinder 2.1 server (Zankari et al., 2012) to identify acquired antimicrobial resistance genes in the genome of each strain.

2.12. Determination of the plasmid origin of E. faecalis bee homolog

To determine the location of the *E. faecalis bee* locus identified in VREr5, plasmid DNA was extracted using QIAprep spin miniprep kit (Qiagen, Hilden, Germany) and Polymerase Chain Reaction (PCR) was performed. Novel primers were designed to detect the *bee*-1 and *bee*-2 genes whereas primers for detection of *bee*-3 gene (P101D12-1, Bee-12) were adopted from Tendolkar et al. (2006).

2.13. Nucleotide accession number

The genome sequences of *E. faecium* strains VRE2, VREr5, VREr6 and VREr7 reported in this study have been deposited in GenBank under accession number LTAA00000000, LTBJ00000000, LTDQ00000000, and LSZZ00000000, respectively.

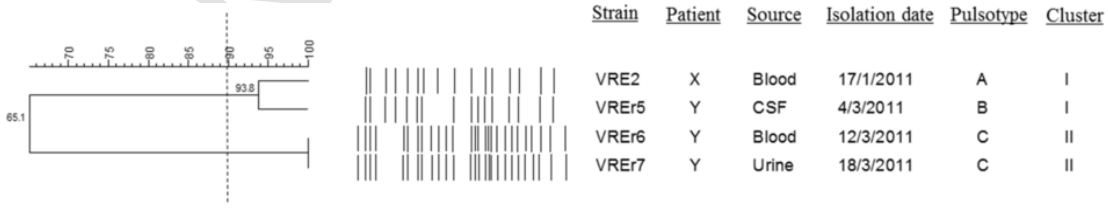
3. Results and discussion

3.1. Clinical background

Patient X was admitted into cardiology ward on 24th October 2010. Subsequently after the isolation of first VRE (VRE2) from this patient (an index case), VRE infection cases were frequently reported in the hospital. Patient X was hospitalized for four months and eventually died on 27th February 2011, approximately one month after patient Y was admitted (21st January 2011). Patient Y was admitted into neuro-ICU ward due to basal ganglia bleed. Microbiological investigation revealed that patient Y was infected by multiple pathogens, including multidrug-resistant *Acinetobacter baumannii*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa*. VREr5 was only isolated from patient Y after one month of treatment with vancomycin. Following this, another two strains, VREr6 and VREr7, were isolated from the same patient who was given an antibiotic treatment with meropenem, colistin, ceftazidime, and linezolid. After the linezolid treatment, VRE was no longer detected in patient Y. To note, patient Y had undergone several surgical procedures such as intracranial pressure monitor and external ventricular drain insertions during hospitalization. These medical procedures could serve as potential routes of bacterial transmission although this association was unclear. Eventually, patient Y died of sepsis due to infective endocarditis and pneumonia.

3.2. PFGE revealed genetic difference of strains isolated from a single patient

PFGE subtyping of the four clinical strains revealed three pulsotypes comprising 16–27 restriction fragments (Fig. 1). Cluster analysis at 90% similarity yielded two clusters. VREr6 and VREr7, which



Strain	Patient	Source	Isolation date	Pulsotype	Cluster
VRE2	X	Blood	17/1/2011	A	I
VREr5	Y	CSF	4/3/2011	B	I
VREr6	Y	Blood	12/3/2011	C	II
VREr7	Y	Urine	18/3/2011	C	II

Fig. 1. Dendrogram showing the cluster analysis of four VRE_{fm} strains based on PFGE patterns of the *Sma*I-digested chromosomal DNA. The dendrogram was constructed using the Dice coefficient and UPGMA clustering parameters at 1.5% position tolerance. The strain code, source, host, and cluster are indicated. The dotted vertical line indicates 90% similarity level.

were isolated from the same patient (patient Y), were indistinguishable (Cluster II). Interestingly, VREr5, which was also isolated from patient Y, was grouped into a different PFGE cluster (cluster I). Instead, VREr5 was closely related to VRE2 (with two bands difference) which was isolated from patient X. These observations indicated that VREr5 is likely to be genetically dissimilar with VREr6 and VREr7, despite being isolated from the same host. The close relationship between VRE2 and VREr5 based on the PFGE results suggested that VREr5 could have derived from the same clone as VRE2.

3.3. General genome signatures and multilocus sequence typing (MLST) of four Malaysian VRE$_{fm}$ strains

To understand the macro-restriction differences observed from the PFGE results, WGS of the four Malaysian strains were performed to dissect their genomes. The general genome features of the four VRE$_{fm}$ strains are summarized in Table 1. The approximate genome sizes of these strains ranged from 2.8 Mbp to 3.0 Mbp. The guanine-cytosine (GC) contents and numbers of predicted protein coding sequences (CDSs) ranged from 37.6% to 37.8% and 2853 to 3057, respectively. Both VRE2 and VREr5 harbored 55 tRNA genes whereas VREr6 and VREr7 harbored 58 tRNA genes.

In silico MLST analysis revealed two sequence types (ST): VRE2 and VREr5 were of ST80 whereas VREr6 and VREr7 were of ST203. Both these sequence types were assigned to the clonal complex 17 (CC17), a specific lineage associated with nosocomial *E. faecium* strains (Top et al., 2008). ST203 is one of the members of the CC17, apart from ST17, ST18 and ST78. In Australia, ST203 has replaced ST17, the founder group of CC17 (Lam et al., 2013). There were 49 entries on ST203 in the PubMLST database (last accessed on 20th Aug 2016) (Homan et al., 2002) and the data showed that this ST is mainly found in European (49%) and Asia Pacific countries (51%). In Malaysia, ST203 was found in isolates recovered from both infected and colonized people (Getachew et al., 2013). In contrast, there were only 13 entries of ST80 in the MLST database which are mainly represented by strains originated from European countries, except for two entries with Asian strains. To the best of our knowledge, this is the first report of ST80 in Malaysia. Strains of CC17 have spread worldwide, including in Malaysia (Getachew et al., 2013; Weng et al., 2013). Our report on ST80 provides new insight into the dissemination of this high-risk clonal complex in Malaysia.

3.4. Comparative genomics revealed variations in genes associated with fitness and adaptive advantage

The genomes of the Malaysian strains were compared with that of *E. faecium* Aus0085 (GenBank accession number CP006620.1), one of the 13 complete *E. faecium* genomes known up to date. Aus0085 was chosen as a reference strain because it shares similar features with our four VRE$_{fm}$ strains. This clinical VRE strain was from similar geographical region, isolated from blood sample of a bacteremia patient (Lam et al., 2013). Moreover, this strain also has the same ST as two of our strains (VREr6, VREr7). Our comparative analyses showed that 2148 open reading frames (ORFs) were shared among all the genomes, which accounted for approximately 71.0% of the total ORFs present in each of the studied strains. This percentage is comparable to that observed by Lam et al. (2013) who compared the conserved ORFs in Aus0085 with other clinical isolates, demonstrating that the core genome of *E. faecium* is stable. This core genome provides evidence of the conservation of genes among *E. faecium* strains. When the four local strains were compared among themselves, the number of core genes increased to 2494. There are 839 dispensable or accessory ORFs whereas the number of strain-specific ORFs ranged from 16 in VRE2 to 57 in VREr5 (Fig. 2). Most of these strain-specific ORFs encode for hypothetical proteins and mobile element proteins, which might play a crucial role in determining distinct virulence features of each strain.

At least four chromosomal regions spanning approximately 169 kbp in Aus0085 showed low or no homology (< 70%) with the four Malaysian strains (Fig. 3). Most of these regions are genomic islands of Aus0085, suggesting that genomic islands can contribute to the genome diversity of *E. faecium*. Most notably, the four studied strains differed from the reference strain by the absence of *vanB*-containing Tn*1549* transposon (Fig. 3). Instead, our VRE$_{fm}$ strains harbored *vanA* gene which is on the Tn*1546* transposon. Differences between these two *van* genotypes will be discussed in the antibiotic section. Furthermore, our results showed that VREr6 and VREr7 shared higher similarity with the reference genome compared to VRE2 and VREr5 (Fig. 3). This observation showed that VREr6 and VREr7 have substantial variations with VRE2 and VREr5. This was further supported by the identification of different genes associated with fitness and adaptive advantage found only in both VRE2 and VREr5 and in both VREr6 and VREr7.

Both VRE2 and VREr5 harbored a cluster of proteins involved in inositol metabolism. Inositol is commonly found in soil and can be used as a sole carbon source in various microorganisms such as *Rhizobium leguminosarum* (Fry et al., 2001) and *Bacillus subtilis* (Yoshida et al., 2008). Van Schaik et al. (2010) reported a 7 kb gene cluster encoding a complete inositol metabolism pathway in the *esp* pathogenicity island (PAI) of another infectious *E. faecium* strain E1679 and demonstrated the capability of this strain to use inositol as a carbon source. This 7 kb gene cluster shared identical amino acid similarity with the gene cluster found in both VRE2 and VREr5. Similar to E1679, this gene cluster was also found integrated into the *esp* PAI of both VRE2 and VREr5. In contrast, homologs of this 7 kb gene cluster were not detected in the genomes of VREr6 and VREr7. The presence of transposases and mobile element proteins in the vicinity of this gene cluster suggested its horizontal origin. Inositol is maintained at a low concentration in blood and urine of healthy people but it is increased in patients with diabetes and diabetic-associated

Table 1
General genome features, sequence types, and clonal complex of the four VRE$_{fm}$ strains.

	VRE2	VREr5	VREr6	VREr7
Genome size (bp)	2,862,609	2,898,367	3,014,993	3,021,201
GC%	37.85	37.8	37.61	37.6
CDS	2853	2906	3049	3057
tRNA	55	55	58	58
rRNA	3	3	3	3
Sequence type	80	80	203	203
Clonal complex	17	17	17	17

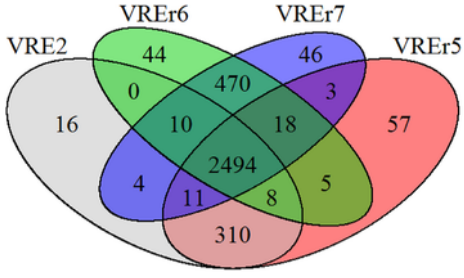


Fig. 2. Venn diagram showing the distribution and number of core, dispensable and strain-specific genes of the Malaysian VRE$_{fm}$ strains. Each circle is labelled with the name of its representative strain. A total of 2494 genes are shared among the analyzed strains. The number of strain-specific genes are 16, 44, 46, and 57 for VRE2, VREr6, VREr7, and VREr5, respectively.

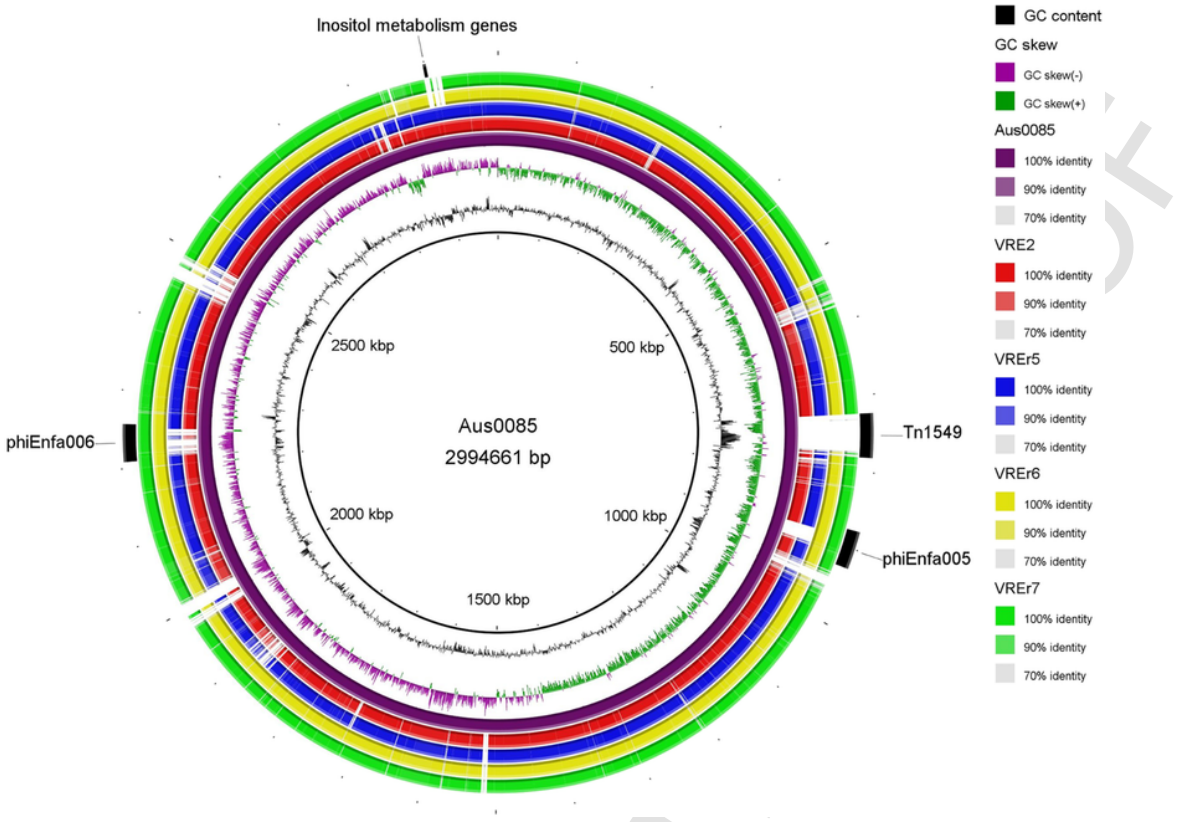


Fig. 3. Circular genomic map and genome comparison of Aus0085, VRE2, VREr5, VREr6, and VREr7. The inner ring shows coordinate in scale and the total genome size of the reference sequence, Aus0085. The black histogram bar represents GC content whereas the purple-green histogram bar represents GC skew. Colored arches representing orthologous regions of each genome in respect to Aus0085 (purple arch) and are shown in the following order (inside to outside): Aus0085, VRE2, VREr5, VREr6, VREr7. The outermost arch (black) represents the location of Tn*1546*, phiEnfa005, phiEnfa006, and inositol catabolism genes relative to Aus0085. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

renal diseases (Hong et al., 2012). The ability to metabolize this sugar and use it as a carbon source may provide an additional growth advantage for these strains to outcompete other bacteria in diabetic patients, such as in patient Y.

It is important to note that the genome sizes of both VREr6 and VREr7 were approximately 140 kb larger than that of VRE2 and VREr5. This size variation was probably contributed by accessory genes which code for phage-related and plasmid-associated proteins. Among the plasmid-associated proteins, an atypical nonlantibiotic bacteriocin, Lactococcin 972 (Lcn972), was identified in VREr6 and VREr7 and shared 100% amino acid sequence identity with that of Aus0085. Bacteriocins are peptides produced by an organism to inhibit the growth of its closely related species (Héchard and Sahl, 2002). Instead of targeting the cytoplasmic membrane and forming pores, Lcn972 inhibits septum formation which leads to deformation and eventually lysis of cells (Martinez et al., 2000). Martinez et al. (2008) reported that this bacteriocin disrupts cell wall biosynthesis by interacting with cell wall precursor lipid II, which is the primary docking site for lantibiotics prior to pore formation. Lcn972 is the first nonlantibiotic that specifically interacts with lipid II. Besides, Lcn972 also plays a role in the induction of prophages, although it is limited to specific prophage/host system (Madera et al., 2009). With its role in killing closely-related strains, the presence of Lcn972 in both VREr6 and VREr7 may increase the competitiveness of these two strains in colonizing and subsequently infecting the host.

3.5. Phylogenomic analysis reveals shared common ancestry of clinical E. faecium and two distinct lineages of the local VRE$_{fm}$ strains

To better understand the relationship among the Malaysian strains and other global strains, a maximum-likelihood tree was constructed based on the core genome alignments of 20 *E. faecium* strains using Aus0085 as the reference (Fig. 4). All these strains, except E0120, are from the CC17. Our results showed that VREr5 was distantly related to VREr6 and VREr7, although they were all isolated from the same host. Instead, VREr5 was more closely related to VRE2 isolated from patient X. This observation is in concordance with the PFGE results. In addition, VREr6 and VREr7 were closely related to strain E161 isolated from China. Notably, these two strains were also more closely related to the reference Aus0085 compared to VRE2 and VREr5, which concurred with the genomic variations discussed in the previous section (Fig. 3). The close relationship observed among our strains and other strains from the CC17 suggested a shared common ancestor among these clinical strains.

Our phylogenomic tree also showed a distinct separation of strains of ST17, ST18, and ST78 into two different clusters. Strains from ST17 (Aus0004, E155, EnGen0180) and ST18 (DO, E1731) were clustered together and were closely related to our Malaysian strains, VRE2 and VREr5. Two strains from ST78 (E6045, E2560) formed another cluster which included VREr6 and VREr7. This observation is consistent with the previous finding that demonstrated the divergence of these three major subgroup founders of CC17 into two dif-

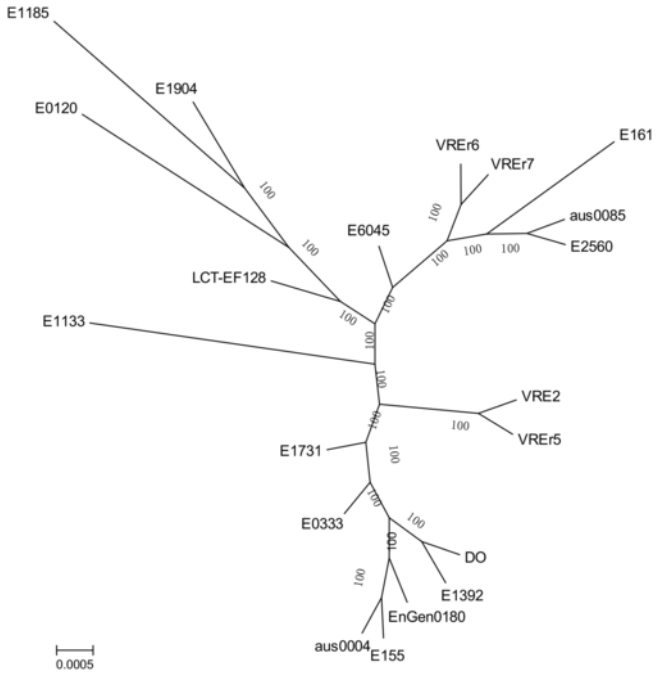


Fig. 4. Phylogenomic tree inferred from approximately-maximum-likelihood method from aligned core genomes. Multiple genomes alignments are generated from 20 global *E. faecium* strains using Aus0085 as a reference. The unrooted phylogenomic tree is inferred via approximately-maximum likelihood method using FastTreeMP (Price et al., 2010). Bootstrap support values are shown in each node.

ferent subgroups using Bayesian Analysis of Population Structure (BAPS) model, with ST78 in BAPS 2-1 and ST17 and ST18 in BAPS group 3-3 (Willems et al., 2012). Due to high recombination rate of *E. faecium*, the inference that CC17 strains originated from a single founder using eBURST algorithm is questionable (Willems et al., 2012). BAPS is an alternative approach to study population structure that accounts for recombination events (Tang et al., 2009). Our results agreed with BAPS analysis which indicated that ST17, ST18, and ST78, which were previously believed to have a same evolutionary history using the eBURST algorithm, were from two evolutionary trajectories which later converged into the same clonal complex through acquisition of genes that characterized CC17 (Willems et al., 2012).

Based on the phylogenomic analysis, together with the differences in PFGE profiles, sequence types and genomic contents, we showed that patient Y was most probably infected by VRE_{fm} strains from more than one lineages. This was supported by retrospective analysis of clinical records which showed that patient Y underwent nasogastric tube exchange between the isolation period of VREr5 and VREr6. This medical procedure might have introduced new VRE_{fm} strains to the patient through contaminated catheters or hands of healthcare workers, hence VREr5, the initial strain isolated from patient Y, was different from the subsequent strains, VREr6 and VREr7. Moreover, since patient Y was admitted when patient X was still in the hospital, the genetic similarity between VRE2 and VREr5 suggested that VRE2 could have persisted and spread in the hospital.

3.6. Mobile genetic elements contribute to genomic plasticity

Several studies have highlighted the roles of mobile genetic elements in defining genome diversity of *E. faecium* strains (Qin et al., 2012; van Schaik et al., 2010). Insertion sequence (IS) elements, prophages, as well as Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) are among the major factors which could contribute to genome dynamics.

A total of 43 to 55 IS elements were identified in our strains. Majority of them were members from the IS*3* (IS*1485*, IS*Efa8*, IS*Efa10*, IS*Enfa3*) and IS*L3* (IS*1251*, IS*Efa11*, IS*1476*, IS*Efa5*) families. IS*16*, which has been suggested as the molecular marker for hospital-associated enterococcus, was also observed (Werner et al., 2011). IS elements can contribute to genome plasticity of bacteria by triggering gene rearrangement, as well as gene inactivation that affects the integration of phages and plasmids (Huh et al., 2004; Ooka et al., 2009). The different types of IS elements identified in the four sequenced strains may have different effects on their genomes, not only in the overall genome structure but also virulence and host adaptation.

Prophages ranging from 39.0 kb to 47.8 kb in size were predicted in the four genomes. Interestingly, high sequence similarity was observed in prophages from strains of the same sequence type. This included two regions in VREr6 and VREr7 which shared 93% to 95% sequence identity with phages phiEnfa005 and phiEnfa006 of Aus0085 (Fig. 3). The ORFs in all the predicted prophage regions mainly contained phage-specific proteins and hypothetical proteins (83.3% to 98.0%). VREr7 shared a similar prophage region with that of VRE2 and VREr5 except for an additional 10.8 kb region containing genes encoding glycopeptide resistance proteins, cadmium transporter, and RelB/RelE toxin-antitoxin system. This region is flanked by two transposases, suggesting that these additional genes were being acquired later by the prophage, indicating that prophages could play an important role in genome diversity of these four strains.

CRISPRs are repetitive sequences that, together with CRISPR-associated (cas) genes, protect bacteria against integration of exogenous DNA such as phages and plasmids into their genomes (Palmer and Gilmore, 2010; Sorek et al., 2008). Qin et al. (2012) reported that clinical *E. faecium* lacks CRISPR loci and this probably explains the higher rate of exogenous DNA in their genomes compared to those of non-clinical *E. faecium* isolates. CRISPR-like regions (designated as questionable CRISPR by CRISPRs Finder) were identified in all four Malaysian strains. However, all these CRISPR-like regions either lacked any *cas* genes or are located within a coding region. Similar results were reported for strains E1071, E1679, and E0371 by van Schaik et al. (2010), who suggested that these partial CRISPR-like loci might not have any functional significance in these strains. The diversity in prophage sequences and abundance of uncharacterized mobile element proteins found in the genomes of our strains might in part due to these possibly non-functional CRISPR regions.

Our molecular subtyping using PFGE, MLST and phylogenomic tree suggested that VREr6 and VREr7 probably came from the same clone. However, the genomic variations contributed by mobile genetic elements allowed us to discriminate these strains. This highlights the discriminatory power of WGS in detecting fine differences of the same clone and hence provides a better resolution in distinguishing strains with high genetic similarity.

3.7. CDS associated with surface polysaccharides, microbial surface components recognizing adhesive matrix molecules (MSCRAMM), and putative virulence factors

The pathogenicity of VRE is contributed by several important virulence factors which are mainly involved in adherence, biofilm formation, invasion and antiphagocytic activity. The virulence associated genes found in the genomes of the four analyzed strains are summarized in Table 2.

Cell-surface polysaccharides are known to play important roles in virulence and phagocytic resistance of Gram-positive bacteria. One such gene cluster, *epa*, was identified in *E. faecalis* and similar gene locus has also been reported in other *E. faecium* (Qin et al., 2012).

Table 2
Selected virulence-associated genes identified in the four Malaysian VRE_{fm} strains.

Gene	Function	Strain			
		VRE2	VREr5	VREr6	VREr7
esp	Biofilm formation	+	+	+	+
acm	Collagen binding	+	+	+	+
efaA	Adherence	+	+	+	+
ebpABC locus	Biofilm-formation	+	+	+	+
bee locus	Biofilm formation	−	+	−	−
pilA (*fms21*)-*fms20* locus	Adherence	+	+	−	−
fms11-19-16 locus	Adherence	+	+	+	+
fms14-17-13	Adherence	+	+	+	+
fms18 (*ecbA*)	Adherence	+	+	+	+
fms15	Adherence	+	+	+	+
bopD	Biofilm formation	+	+	+	+
epa operon	Biosynthesis of cell-surface polysaccharides	+	+	+	+
cpsA	Antiphagocytosis	+	+	+	+
cpsB	Antiphagocytosis	+	+	+	+

"+" indicates the presence of virulence genes whereas "-" indicates the absence of virulence genes.

The *epa* gene cluster encodes for proteins involved in the biosynthesis of an antigenic cell wall polysaccharide composed of rhamnose, glucose, galactose, *N*-acetylgalactosamine and *N*-acetylglucosamine (Teng et al., 2009). Disruption of the *epa* locus showed attenuation in biofilm formation, phagocytic resistance and tissue invasion in *E. faecalis* (Teng et al., 2009; Zeng et al., 2004). The *epa* gene cluster of *E. faecalis* consists of 18 genes (*epaA* to *epaR*) but only 15 of them (*epaA-H*, *epaL-M*, *epaO-R*) are found in *E. faecium* (Qin et al., 2012). Besides, these 15 genes are ordered differently as those found in *E. faecalis* (Qin et al., 2012). All the 15 *epa* genes were present in our four VRE_{fm} strains, sharing same organization and high amino acid identities (88–100%) with that of the reference strain Aus0085. Qin et al. (2012) had reported the conservation of this gene cluster between TX16 and 21 *E. faecium* draft genomes. Our comparative genome analyses on *epa* gene cluster of TX16 further confirmed that these genes are part of the core genome of *E. faecium*. The virulence nature as well as the conservation of these *epa* genes in *E. faecium* suggests that these genes can serve as a potential target for new drug development against infections caused by this pathogen.

Surface expressed proteins such as pili and MSCRAMM are important virulence factors involved in adhesion, biofilm formation, and invasion of Gram-positive bacteria. A total of 15 genes encoding cell-wall anchored proteins with MSCRAMM features were previously identified in *E. faecium* TX16 (Nallapareddy et al., 2003; Sillanpää et al., 2009, 2008). Eleven of these genes form four gene clusters while the rest are present as a single gene. Three gene clusters, *ebpABC*, *fms11-fms19-fms16*, and *fms14-fms17-fms13*, were found in the genomes of all four local strains. Among these, the *ebpABC* cluster, which encodes for biofilm-associated pili, was shown to be important in urinary tract infection in an animal model (Nallapareddy et al., 2011). Another gene cluster, *fms21-fms20*, was detected only in VRE2 and VREr5 but not in VREr6 and VREr7. The *fms21-fms20* cluster is carried on a transferable plasmid (Kim et al., 2010), suggesting that VRE2 and VREr5 acquired these genes horizontally. All the four analyzed strains harbored *acm*, encoding the collagen binding protein Acm, which plays a significant role in endocarditis (Nallapareddy et al., 2008). Another collagen-binding protein, Fms18 (*fms18*) was also found in our strains. In contrast, the *scm* gene which encodes the second collagen-binding protein in *E. faecium*, was absent in all our strains.

Our search for enterococcal virulence factors using the virulence factor database (VFDB) also detected homologs of endocarditis specific antigen EfaA (63% amino acid identity) and a sugar transcriptional regulator BopD (87% amino acid identity) of *E. faecalis* in the genomes of the four studied strains. Two capsule-associate genes (*cpsA*, *cpsB*) were also found in all the studied genomes, which may protect the strains from host phagocytic activities. Additionally, the enterococcal surface protein (Esp), which is the hallmark in clinical isolates, was also identified in these strains. Other major virulence factors listed in the VFDB such as gelatinase and hyaluronidase were not detected in any of the four studied strains.

3.8. Homologs of E. faecalis bee locus revealed possible association with biofilm formation in VREr5

Biofilm formation is an important virulence factor of enterococci as these organisms are frequently isolated from biofilm-mediated infections such as endocarditis and those associated with indwelling medical devices (Donlan et al., 2002). Furthermore, there is a potential transfer of enterococcal antibiotic resistance genes to other more pathogenic bacteria such as *Staphylococcus aureus* in mixed-species biofilm as the horizontal transfer rate of antimicrobial resistance genes is high in this type of biofilm (Donlan et al., 2002). Hence, crystal violet assay was performed to investigate the biofilm forming ability of the four strains.

Based on the interpretation scheme adopted from Stepanović et al. (2000), our results showed that VREr5 was a strong biofilm producer whereas the rest of the strains were non-biofilm producers (Table 3). To confirm these results, CLSM was performed. The acquired CLSM images showed that VREr5 formed dense biofilm (Fig. 5). In contrast, VRE2 and VREr6 can only form small aggregates. VREr7, on the other hand, produced a thin monolayer on the chamber slide. The CLSM images confirmed the biofilm forming ability of VREr5. However, VREr7, which was classified as non-biofilm producer based on the crystal violet assay, showed potential biofilm forming ability as observed from the CLSM image. One of the possible explanations for these observations is the different abiotic surfaces used to grow biofilm in the crystal violet assay and for CLSM imaging. The effect of different abiotic substrates on bacterial biofilm formation had been demonstrated in other study (Tendolkar et al., 2004). Since VREr5 was the only strong biofilm former, we examined the genome of VREr5 and found five genes which resembled the *bee* locus of *E. faecalis*.

The cut-off OD (ODc) was defined as three standard deviations above of the mean OD of the negative control. The biofilm forming ability of each strain was scored as follow: $OD \leq ODc$ = non-biofilm producer, $ODc < OD \leq (2 \times ODc)$ = weak-biofilm producer, $(2 \times ODc) < OD \leq (4 \times ODc)$ = moderate-biofilm producer, $OD > (4 \times ODc)$ = strong-biofilm producer (Chelvam et al., 2014; Stepanović et al., 2000).

The *bee* locus (*b*iofilm *e*nhancer in *e*nterococcus) is a cluster of biofilm-associated genes composed of three genes (*bee*-1, *bee*-2, *bee*-3) encoding putative cell wall-anchor proteins and two genes (*str*1, *str*2) encoding for sortases (Tendolkar et al., 2006). Insertion of

Table 3
Average results of three replicates of the crystal violet assay to determine biofilm forming potential.

Strain	OD_{590}	Interpretation[a]
VRE2	0.016	Non-biofilm producer
VREr5	1.780	Strong-biofilm producer
VREr6	0.049	Non-biofilm producer
VREr7	0.083	Non-biofilm producer

[a] Interpretation based on ODc of 0.218.

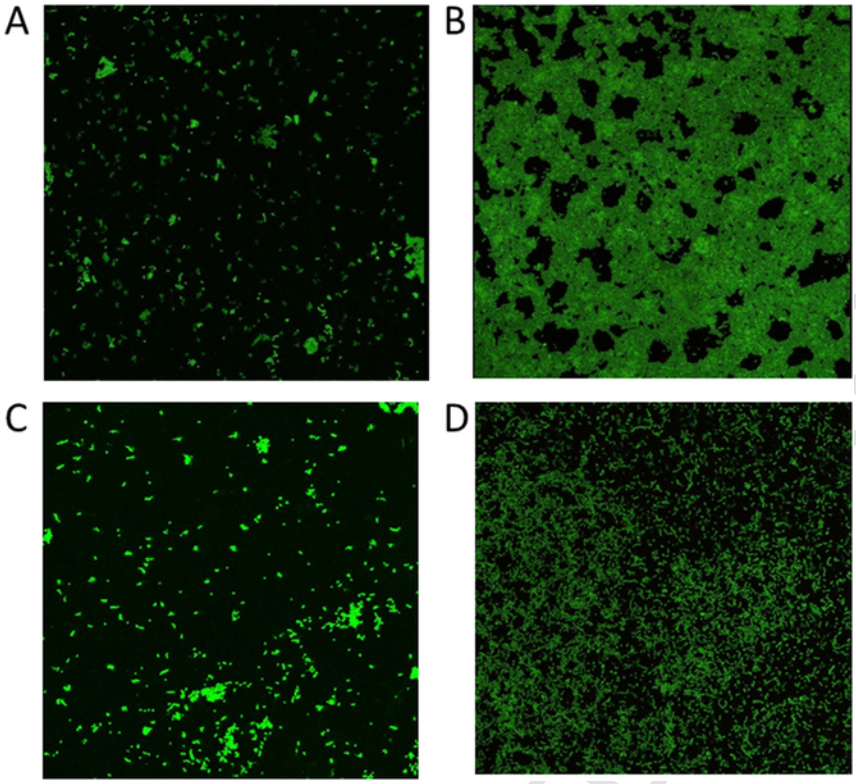


Fig. 5. CLSM images of the four Malaysian VRE_{fm} strains grown in TSB. Each image represents the "flatten" three-dimensional (3D) Z-projection of stack images of A) VRE2, B) VREr5, C) VREr6, and D) VREr7. The non-biofilm formers (VRE2, VREr6) were either scattered around or formed small aggregates on the glass slide. VREr7 formed thin monolayer whereas VREr5 formed dense biofilm.

Tn*917* within *bee-2* has led to a 70% reduction of biofilm formation in *E. faecalis* strain E99 (Tendolkar et al., 2006). Four of the five unique genes of VREr5 showed high amino acid identity (99%) with Bee-2, Bee-3 and the two sortase proteins of the *bee* locus whereas one of the unique genes showed only 39% amino acid identity with Bee-1. Despite its low amino acid similarity, the putative Bee-1 homolog of VREr5 shared similar structures of the Bee-1 protein. These included a region from residues 705 to 810 that showed low degree of similarity (E = 1.08e-03) to collagen binding B domain of *Staphylococcus aureus*, and a region from 338 to 469 that encodes for the Von Willebrand factor type A (VWA) domain. The VWA domain is usually associated with pilus and can bind to a variety of ligands, including collagen, laminin and human epithelial cells (Konto-Ghiorghi et al., 2009; Whittaker and Hynes, 2002). Given the high similarity of the unique gene cluster of VREr5 to that of *bee* locus and being the only strong biofilm former, an association of the *bee* locus homolog to biofilm formation cannot be ruled out. However, further analyses are needed to confirm this association.

The *E. faecalis bee* locus has been reported to be located on a large conjugative plasmid (Coburn et al., 2010). To determine the location of the *bee* homolog in the VREr5, plasmid DNA was extracted and PCR using the primers targeting *bee*-1, *bee*-2 and *bee*-3 was performed. Our PCR results showed that all these three genes were successfully amplified (Data not shown), suggesting that the *bee* locus homolog was most probably located on a plasmid.

3.9. Association between antibiotic resistance phenotypes and genotypes

Enterococci, particularly *E. faecium*, are resistant to multiple antibiotics commonly use in treatment of enterococcal infections. This resistance can be intrinsic or acquired through mutation of the intrinsic genes or acquisition of mobile elements carrying the resistant determinants. The antibiotic susceptibility profile of the four sequenced strains is shown in Table 4. All these strains were resistant to vancomycin, ampicillin, kanamycin, streptomycin, erythromycin, clindamycin and tetracycline. VRE2 was susceptible to gentamicin, whereas the other three strains were resistant to gentamicin. VRE2 and VREr5 showed intermediate resistance to teicoplanin whereas VREr6 and VREr7 were resistant to it. None of the sequenced strains was resistant to linezolid.

Based on the microbiological data from hospital records, no VRE strain was isolated from patient X and patient Y at the time of admission. Within three months, VRE2 was isolated from patient X. For patient Y, VREr5 was isolated after one month of admission and vancomycin treatment. These observations suggested that patient X and patient Y could have acquired VRE strains due to their prolonged hospitalization, or that vancomycin resistance of the colonized VRE was induced after vancomycin was prescribed.

Both *vanA* and *vanB* are the major determinants of acquired vancomycin resistance in enterococci. Our four strains carried the *vanA* gene whereas the reference strain Aus0085 harbors *vanB* gene. Although these two resistance genotypes are widely distributed, B-type resistance is mainly reported in Australia and Singapore, while A-type resistance is predominantly found in the United States, Europe, and Korea (Bonten et al., 2001; Lam et al., 2013; Molton et al., 2013; Yoo et al., 2006). In Malaysia, *vanA* genotype has been reported (Mohamed et al., 2015). VanA phenotype is characterized by high level of inducible resistance to both vancomycin and teicoplanin whereas VanB phenotype shows inducible resistance to vancomycin but susceptible to teicoplanin (Arthur and Courvalinn, 1993; Hollenbeck and Rice, 2012). In this study, VRE2 and VREr5 had

Table 4
Antimicrobial resistance profile and the presence of corresponding resistance genes.

	VAN		TEC		KAN			GEN		STM		AMP		ERY		CLI		TET			LZD	
	R/I/S	*vanA*	R/I/S	*vanZ*	R/I/S	*aac(6′)-Ii*	*aph(3′)-III*	R/I/S	aac(6′)-aph(2″)	R/I/S	*ant(6)-Ia/aadE*	R/I/S	Mutated *pbp5*	R/I/S	*ermB*	R/I/S	*msrC*	R/I/S	*tetS*	*tetM*	R/I/S	G2576T mutation in 23S rRNA
VRE2	R	+	I	+	R	+	+	S	−	R	+	R	+	R	+	R	+	R	+	−	R	−
VREr5	R	+	I	+	R	+	+	R	+	R	+	R	+	R	+	R	+	R	+	−	R	−
VREr6	R	+	R	+	R	+	+	R	+	R	+	R	+	R	+	R	+	R	−	+	R	−
VREr7	R	+	R	+	R	+	+	R	+	R	+	R	+	R	+	R	+	R	−	+	R	−

Resistance gene(s) or mutation associated with each antibiotic is (are) listed below the abbreviations of respective antibiotics. VAN: vancomycin, TEC: teicoplanin, KAN: kanamycin, GEN: gentamicin, STM: streptomycin, AMP: ampicillin, ERY: erythromycin, CLI: clindamycin, TET: tetracycline, LZD: linezolid. Plus (+) and minus (−) signs indicate presence and absence of the resistance genes or mutation, respectively. The R/I/S column gives information regarding the susceptibility of each strain to respective antibiotics: R = resistant, I = intermediate, S = sensitive.

VanA phenotype (vancomycin MIC > 256 μg/ml, teicoplanin MIC = 16 μg/ml) whereas VREr6 and VREr7 displayed VanB phenotype-*vanA* genotype, which were highly resistant to vancomycin (MIC > 256 μg/ml) but susceptible (MIC = 8 μg/ml) to teicoplanin. The occurrence of VanB phenotype-*vanA* genotype isolates highlights the importance of MIC determination for *vanA* VRE in establishing the resistance phenotype and choice of treatment methods against this pathogen. It has been reported that point mutations of *vanS* or disruption of *vanY* or *vanZ* lead to impaired teicoplanin resistance in *vanA* genotype VRE strains (Gu et al., 2009; Hashimoto et al., 2000). However, no mutations or disruptions of these regions were observed in VREr6 and VREr7. Further analysis is needed to identify the cause of this impairment.

The *vanA*-associated Tn*1546* transposon is polymorphic (Gagnon et al., 2011; Huh et al., 2004; Willems et al., 1999). Most of the known structural variations are caused by IS elements, which usually lead to complete or partial loss of the *vanA* cluster elements (Huh et al., 2004). Our detailed structural analysis revealed that the Tn*1546*-like transposons of the four studied strains were similar. The left end of the transposon, encoding a transposase (orf1) was truncated, with one IS*1216V* insertion directly before *orf2*. Moreover, an IS*1251* was found inserted in the intergenic region of *vanS* and *vanH*. The structure observed resembled type F reported by Willems et al. (1999). Insertion of IS elements may change the level of vancomycin resistance by disrupting the *van* elements, particularly through partial or complete deletion of *vanR* and *vanS* (Gagnon et al., 2011). However, we could not verify the effects of IS insertions as all our strains had the same MIC (≥ 256 μg/ml).

Aminoglycoside resistance is mediated by three types of aminoglycoside modifying enzymes: N-Acetyltransferases (AAC), O-Adenyltransferases (ANT), and O-phosphotransferases (APH) (Hollenbeck and Rice, 2012). Three genes of these enzymes, *ant(6)-Ia*, *aac(6')-Ii* and *aph(3')-III* were found in all four VRE_{fm} strains, which could confer resistance to streptomycin, tobramycin, and kanamycin, respectively. The *ant(6)-Ia* (also known as *aadE*) gene is often found in a cluster of *ant(6)-sat4-aphA*, which confers resistance to streptomycin, streptothricin, and kanamycin. This resistance gene cluster was identified in all four studied strains, sharing 100% nucleotide identity with the published sequence reported by Werner et al. (2001) (GenBank accession no. AF330699). Additionally, a bifunctional gene, *aac(6')-aph(2")*, which confers high-level acquired resistance to gentamicin was identified in the Malaysian strains, except for VRE2. The absence of this resistance gene might explain the susceptibility of VRE2 towards gentamicin (M.I.C = 4 μg/ml). The rest of the three strains showed high-level resistance to gentamicin (M.I.C > 256 μg/ml). Gentamicin is one of the recommended aminoglycosides used in combination with a cell wall active agent in the synergistic therapy of severe enterococcal infections (Hollenbeck and Rice, 2012). High-level resistance to gentamicin eliminates the synergistic killing effect, further challenging the development of new treatment methods against enterococcal diseases.

E. faecium is intrinsically resistant to β-lactam drugs through the expression of low-affinity penicillin-binding protein 5 (PBP5). Increased resistance to β-lactam drugs can be developed through the acquisition of β-lactamase or *pbp5* mutation. Four β-lactamases were identified in each of the studied strains. These included two from metallo-β-lactamase superfamily, one putative class C β-lactamase and one from unknown-class β-lactamase. However, high- level β-lactam resistance in *E. faecium* is commonly contributed by mutations in PBP5. In our study, three types of point mutations were observed in the *pbp5* genes of the four sequenced strains: insertion of a serine residue at position 466, after a serine residue; replacement of methionine at position 485 to alanine and replacement of glutamic acid at position 629 by valine. All these mutations are associated with increased MICs of β-lactam drugs (Rice et al., 2004).

The four strains were also resistant to erythromycin, which was conferred by the *ermB* gene. Additionally, all the studied strains carried the *msrC* gene, a homolog of staphylococcal *msrA*, which encodes an ABC efflux pump for macrolides and streptogramin B (Singh et al., 2001). Two types of tetracycline resistance genes were observed among the four studied strains. VRE2 and VREr5 harbored the *tetS* gene whereas VREr6 and VREr7 carried the *tetM* gene. Both these genes confer tetracycline resistance through ribosomal protection and are mostly located on conjugative transposon or self-transferrable plasmids (Charpentier et al., 1993; Martin et al., 1986).

Overall, our data showed high similarity in antibiotic resistance profile in the three strains isolated from patient Y, demonstrating that no additional resistance determinants were acquired or induced during the isolation period. There was a concordance in the observed antibiotic resistance phenotypes and the presence of the respective resistance genes or mutations

4. Conclusions

To the best of our knowledge, this is the first comparative genomic analysis on the Malaysian VRE_{fm}. Four clinical VRE_{fm} strains from two fatal cases were shown to be different based on the PFGE analysis, particularly three strains from a single patient (patient Y). Detailed genome analyses revealed that VREr5, the initial isolate from patient Y, was genetically more similar to that of VRE2 isolated from an index case (patient X). Molecular analyses including MLST and phylogenomics further indicated that VREr5 was different from the other two strains (VREr6, VREr7) isolated from patient Y. Coupled with clinical data, these results revealed that patient Y was infected with multiple strains of VRE_{fm} from different clones. Additionally, the first isolated strain from patient Y was probably of the same clone as the strain from the index case, patient X. Our study also detected a number of antibiotic resistance- and virulence genes in the four VRE genomes which could contribute to their persistence and pathogenicity. Furthermore, there is a possible association of the homolog of *E. faecalis bee* locus with the potential to form biofilm. This is the first report on the emergence of ST80 VRE strains in Malaysia.

Overall, our molecular analyses have shed light on the genetic relatedness of the VRE_{fm} strains isolated from the two clinical cases, as well as the virulence nature of these strains. Future study to include a bigger sample size of the local strains should be considered to provide a better overview on the genome diversity of VRE_{fm} in Malaysia.

Uncited reference

Derbise et al., 1997

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