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Research Note

A Report of Vancomycin-susceptible, Teicoplanin-resistant *Enterococcus faecalis* ST6 in Malaysia

Loong, S.K.[a], Che Mat Seri, N.A.A.[a], Mahfodz, N.H.[a], Ahmad Nasrah, S.N.[b], Akbar, S.Z.[a] and AbuBakar, S.[a,b*]

[a]Tropical Infectious Diseases Research & Education Centre (TIDREC), Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

[b]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

[*]Corresponding author e-mail: sazaly@um.edu.my

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Abstract. The ability to acquire antibiotic resistance and virulence has propelled *Enterococcus faecalis* to become a major nosocomial pathogen. In Malaysia, data on the antibiotic resistance determinants and virulence of *Enterococcus* circulating strains are still scarce. This study aimed to assess the genotype of an *E. faecalis* isolate initially identified as *Streptococcus uberis*, examine the antibiotic resistance genotypes, analyze the genetic variations within Tn*1546* and investigate the presence of virulence genes. The *E. faecalis* isolate was genetically characterized using multilocus sequence typing (MLST). Minimum inhibitory concentrations to vancomycin and teicoplanin were determined. Antibiotic resistance and other virulence genes were amplified using nucleic acid polymerase chain reaction. Mapping of the Tn*1546* transposon was performed and compared to the prototype sequences. The *E. faecalis* isolate was found to have a MLST profile corresponding to sequence type 6. The isolate was resistant to teicoplanin but susceptible to vancomycin. Its genome consisted the *vanA* and *vanC1* genes. Novel genetic variations in the *vanS*, *vanS-vanH* intergenic region and *vanY* genes were present and six virulence genes were detected. The detection of the *vanC1* gene, thought to be non-transferable, suggests the potential emergence of inter-species enterococcal *vanC1* gene transfer. The peculiar antibiotics resistance phenotype of this *E. faecalis* isolate could be associated to the novel genetic variations found. This study highlights the presence of *E. faecalis* belonging to the high-risk clonal complex with multiple virulence factors in Malaysia.

Enterococcus faecalis was classically considered as a commensal of the human gastrointestinal tract. More recently, the bacteria has become a clinically relevant pathogen involved in hospital-acquired bloodstream, urinary tract and surgical wound infections (Penas *et al.*, 2013). This bacterium is now known to cause severe disease in humans and is becoming a major threat due to its inherent ability to acquire antibiotic resistance (Eaton & Gasson, 2001). Acquired antibiotic resistance coupled with the natural tolerance of these organisms to harsh environmental conditions (low pH, high salt concentrations and high temperatures) may confer selection advantage over other bacteria species (Eaton & Gasson, 2001; Kayaoglu & Ørstavik, 2004). The Enterococci *vanA* gene is induced by the glycopeptides, vancomycin and teicoplanin and this resistance determinant is encoded on the transposon Tn*1546* (Hashimoto *et al.*, 2000). Mapping the transposable elements could shed important information about the

mechanism of resistance. Additionally, enterococci possess virulence factors that participate in the establishment of infection and survival in the host (Eaton & Gasson, 2001; Kayaoglu & Ørstavik, 2004; Penas *et al*., 2013).

In Malaysia, almost 4 000 cases of enterococcal infections were reported between 2006 and 2007 (Weng *et al*., 2013). Data on the glycopeptide resistance determinants and virulence of *Enterococcus* circulating strains are however, still scarce. The advancement of molecular epidemiological methods has contributed to the gain in knowledge on the population structure of enterococcal species isolated from humans (Freitas *et al*., 2009). The aims of the current study were to assess the genotype of an *E. faecalis* isolate initially identified as *Streptococcus uberis* by multilocus sequence typing (MLST), examine the antibiotic resistance genotypes, analyze the genetic variations within Tn*1546* and investigate the presence of virulence genes. These information may provide insights into the genetic evolution of *E. faecalis* and help to understand the complex pathogenic processes involving this bacterium.

In our repository, we discovered one isolate of *E. faecalis* isolated in July 2011 from the urine of a patient. This isolate was initially identified as *S. uberis* using API 20 Strep (bioMérieux, Marcy l'Etoile, France). Due to its suspicious potential acquisition since *S. uberis* is the etiological agent of bovine mastitis (Mian *et al*., 2002) and it is uncommon in an urban setting, 16S rDNA sequencing (Misbah *et al*., 2005) was performed for further confirmation. The resulting 16S rDNA sequences identified this bacterium as *E. faecalis*. MLST and multiplex PCR to determine the presence of vancomycin resistance genes were performed according to published protocols (Dutka-Malen *et al*., 1995; Ruiz-Garbajosa *et al*., 2006). Minimum inhibitory concentrations (MICs) to vancomycin and teicoplanin were determined using M.I.C. Evaluator Strips (Thermo Fisher Scientific, Cheshire, UK) on Mueller-Hinton agar incubated at 35±2°C under ambient conditions for 24 h. The interpretation of antibiotic resistance, intermediate resistance or susceptibility was performed according to the Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute, 2014). MIC assays were repeated once to verify the earlier findings. Other structural elements of Tn*1546*, the transposon carrying the *vanA* operon were examined using overlapping nucleic acid polymerase chain reaction (PCR) amplification targeting its internal region (Huh *et al*., 2004). Sizes and nucleotide sequences of all PCR fragments were compared against the prototype Tn*1546* of *E. faecium* strain BM4147. The presence of virulence genes (collagen-binding protein; *ace*, aggregation substance; *asa*, cytolysin activator; *cylA*, endocarditis antigen; *efaA*, extracellular surface protein; *esp* and gelatinase; *gelE*) was determined using PCR amplification of respective genes (Sedgley *et al*., 2005). Amplified DNA fragments were sequenced and compared against sequences available in the GenBank.

MLST of the *E. faecalis* isolate resulted in a profile corresponding to sequence type 6 (ST6). Resistance to teicoplanin at 64 mg/ml was demonstrated using MIC assay, however it was vancomycin-sensitive. Structural comparison of the Tn*1546* between the Malaysian isolate and the prototype revealed that both carried similar sized amplified DNA fragments and they were confirmed by sequencing. Subsequent analyses into the nucleotide sequences exposed point mutations in the *vanS*, *vanY* and *vanS-vanH* intergenic region, and a deletion in the *vanY* (Figure 1A-C). The *vanS* contained point mutations at positions 148 (T to G), 160 (G to C), 207 (A to T) and 527 (A to T) (Figure 1A), resulting in the amino acid substitutions L148V, E160Q, Q207H and Y527F. The *vanY* contained a point mutation at position 229 (G to C) (Figure 1C). The *vanS-vanH* intergenic region contained one point mutation at position 77 (G to A) (Figure 1B) and a deletion was observed at position 20 in *vanY* (Figure 1C). Vancomycin resistant genes; *vanA* and *vanC1*, and the virulence genes; *ace*, *asa*, *cylA*, *efaA*, *esp* and *gelE*, were

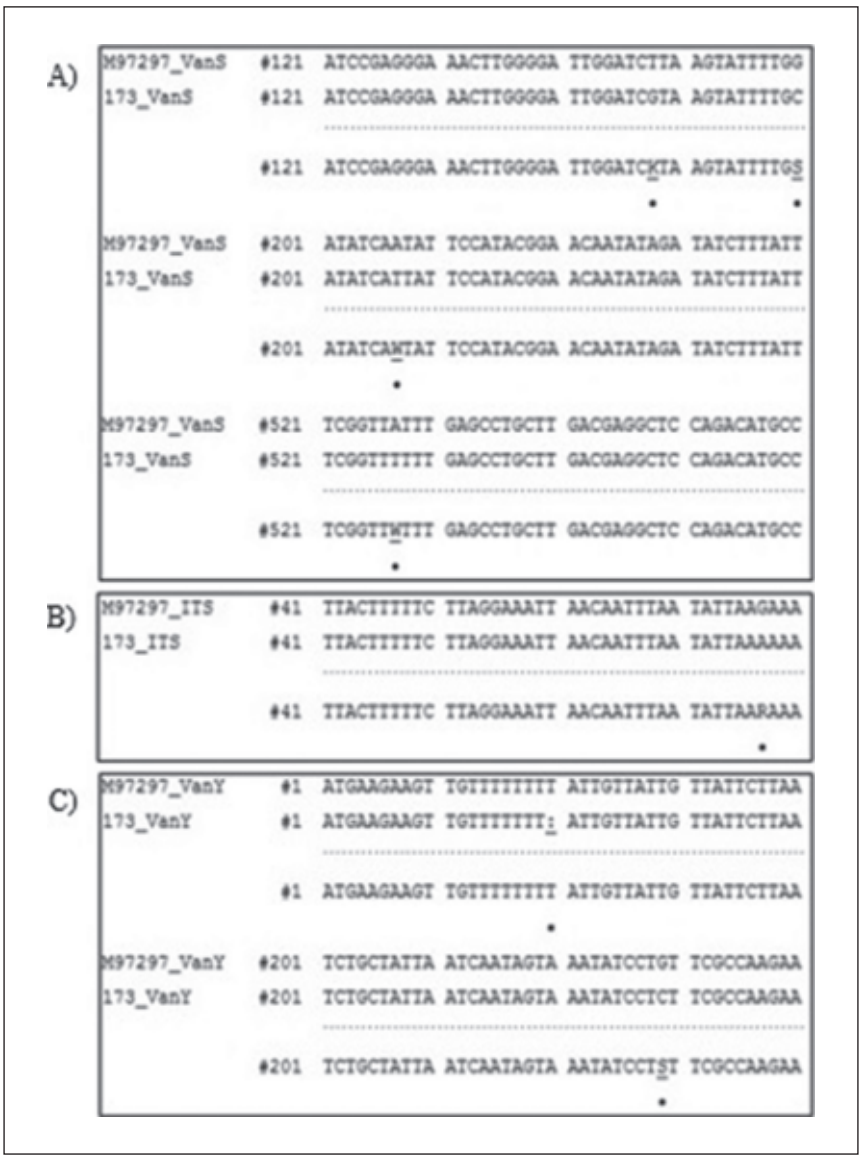

Figure 1. Nucleotide sequence alignments of (A) *vanS*, (B) *vanS-vanH* intergenic region and (C) *vanY* nucleotide sequences. Dissimilar residues are indicated by underlining of the consensus sequence and solid black dots below them. The deletion is indicated by an underline at the particular point of deletion and a solid black dot below the consensus sequence. The prototype, *E. faecium* strain BM4147 is indicated by its Tn*1546* accession number, M97297 and the Malaysian *E. faecalis* is indicated by the isolate number, 173. Corresponding genes (*vanS* and *vanY*) and intergenic region (ITS) are indicated after the names of the studied isolates.

amplified from this isolate (Figure 2). The accession numbers of the sequences reported in this paper are LN829115 (16S rDNA), LN829116 (*vanA*), LN829117 (*vanC1*), LN868269 (*vanS*), LN868270 (*vanY*) and LN868271 (*vanS-vanH* intergenic region).

E. faecalis ST6 has been previously discovered among hospital isolates in an earlier study on *Enterococcus* epidemiology in Malaysia (Weng *et al.*, 2013). That study however, did not investigate the presence of vancomycin-resistant and virulence genes

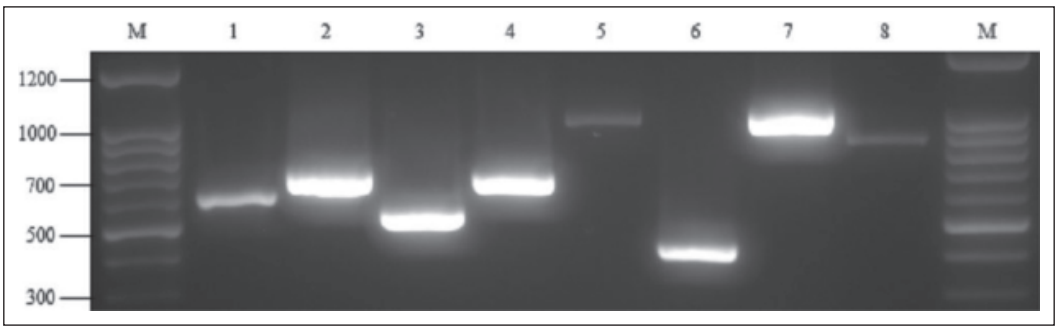

Figure 2. Nucleic acid amplification of virulence and vancomycin-resistant genes of *Enterococcus faecalis* ST6 from Malaysia. M, molecular size marker (in bp); Lane 1. *ace* (616 bp); Lane 2. *efaA* (688 bp); Lane 3. *asa* (529 bp); Lane 4. *cylA* (688 bp); Lane 5. *esp* (932 bp); Lane 6. *gelE* (405 bp); Lane 7. *vanA* (1030 bp); Lane 8. *vanC1* (822 bp).

among enterococci, and their *E. faecalis* ST6 isolate was vancomycin and teicoplanin-sensitive. ST6 isolates belong to the high risk clonal complex 2 (CC2) which includes isolates that cause hospital epidemics and are disseminated worldwide (Freitas *et al.*, 2009). The discovery of a similar *E. faecalis* CC2 in a different Malaysian hospital (Weng *et al.*, 2013) suggests the existing pressure of this lineage in Malaysia. To the best of our knowledge, this is the first report of an *E. faecalis* CC2 harboring the *vanC1* gene. Previous reports on the detection of *vanC1* gene in *E. faecalis* were all from animal isolated strains (de Garnica *et al.*, 2013). This is an important finding with regards to species identification because *vanC1* has been established as a species marker of *E. gallinarum* (Dutka-Malen *et al.*, 1995). As hospital infections caused by *E. gallinarum* have been reported (Praharaj *et al.*, 2013), it is possible that our isolate may have acquired the *vanC1* gene via horizontal transfer from hospital strains. Another explanation for this finding could be that horizontal transfer of *vanC1* by environmental or animal strains occurred prior to them entering the hospital, as attested by the detection of *E. gallinarum* in local broilers (Getachew *et al.*, 2009). Inter-host transmission between hospital-acquired human and animal strains has been suggested in several European countries (Freitas *et al.*, 2011), making this explanation even more plausible.

Resistance to vancomycin has been demonstrated to be regulated by the VanR-VanS two component regulatory system which activates a promoter for the co-transcription of *vanH*, *vanA* and *vanX* genes (Huh *et al.*, 2004). In our isolate, a point mutation was observed in the putative promoter region located in the *vanS-vanH* intergenic region (Huh *et al.*, 2004). As a result, transcription was possibly affected, resulting in the deterioration of vancomycin resistance. The *vanY* encodes accessory proteins which contribute moderately to vancomycin resistance (Arthur *et al.*, 1994). The point deletion and mutation observed in our isolate probably acted in tandem to exacerbate the deterioration of vancomycin resistance by interrupting protein synthesis. Previous reports have implicated three amino acid substitutions in *vanS* (L148V, E160Q and Q207H) in the impairment of teicoplanin resistance in Enterococci strains (Hashimoto *et al.*, 2000). Our isolate which is clearly teicoplanin resistant (MIC, 64 mg/ml), carried an additional substitution (Y527F) whose role has not been ascertained but could possibly negate the impairment process. Taken together, the peculiar phenotype of vancomycin-susceptible and teicoplanin-resistant could be associated to the novel genetic variations found in the Tn*1546* elements.

Despite enterococci being important opportunistic pathogens, very little is known about the virulence mechanisms that contribute to pathogenesis. Enterococci possess conjugative plasmids that can be utilized for the transfer of virulence and

antibiotic resistant genes (Eaton & Gasson, 2001; Kayaoglu & Ørstavik, 2004; Ruiz-Garbajosa *et al*., 2006). Using previously published genome sequence amplification protocols (Eaton & Gasson, 2001; Sedgley *et al*., 2005), we detected the presence of six virulence genes in our isolate. These include collagen-binding protein, aggregation substance, cytolysin activator, endocarditis antigen, extracellular surface protein and gelatinase. *Ace*, the collagen-binding microbial surface component recognizing adhesive matrix molecules (MSCRAMM) is involved in adhesion of *E. faecalis* to host cells and tolerance to stressful growth conditions (Kayaoglu & Ørstavik, 2004; Sedgley *et al*., 2005). *Asa*, which also has adhesive function, is involved in the protection of *E. faecalis* against host defense mechanisms (Kayaoglu & Ørstavik, 2004). Another surface adhesin, *efaA* was hypothesized as an endocarditis marker and shown to be essential for the growth and survival of *E. faecalis* in ion depleted environments (Eaton & Gasson, 2001; Kayaoglu & Ørstavik, 2004). The enterococcal extracellular surface protein, *esp* is involved in *E. faecalis* colonization of the urinary tract and immune evasion (Eaton & Gasson, 2001; Kayaoglu & Ørstavik, 2004; Penas *et al*., 2013). Gelatinase and cytolysin activator have tissue damaging functions (Eaton & Gasson, 2001; Kayaoglu & Ørstavik, 2004) with the former suggested to provide nutrients to *E. faecalis* by hydrolyzing host proteins (Eaton & Gasson, 2001; Kayaoglu & Ørstavik, 2004; Sedgley *et al*., 2005) and the latter involved in the inhibition of other bacteria (Kayaoglu & Ørstavik, 2004; Penas *et al*., 2013). The presence of the virulence genes described above suggests that our *E. faecalis* isolate has the necessary requirements to establish and maintain infection that could be destructive to the human host.

This clinical isolate which also harbors the *vanC1* gene previously thought to be non-transferable (de Garnica *et al*., 2013; Praharaj *et al*., 2013), highlights the emergence of inter-species Enteroccocal gene transfer and the discovery of genetic variations suggests that dissemination of resistant genes occurred through transposable elements, Tn*1546* and not by clonal dissemination. Increased surveillance into the prevalence of other enterococci infections in the hospital is important to control the spread of these organisms, especially considering that our isolate belonged to the high-risk clonal complex adapted to the hospital environment.

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