ORIGINAL PAPERS

Adv Clin Exp Med 2015, **24**, 1, 121–127
DOI: 10.17219/acem/38162

ISSN 1899–5276

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Genotypic Variations of Virulent Genes in *Enterococcus faecium* and *Enterococcus faecalis* Isolated from Three Hospitals in Malaysia*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. The genus *Enterococcus* is of increasing significance as a cause of nosocomial infections, and this trend is exacerbated by the development of antibiotic resistance.
Objectives. The aim of the present study was to estimate the potential virulence factors in *enterococci* and to ascertain their prevalence in Malaysian hospitals.
Material and Methods. The study comprised 222 enterococcal strains isolated from blood, urine, exudates, sputum, stool and body fluid. These strains were collected from patients staying in three referral hospitals in Malaysia. All isolates were identified to the species level, and their MIC of vancomycin was determined using E test strips. Specific primers were designed for detection of the five potential virulence genes (*gelE*, *PAI*, *esp*, *ace*, and *sprE*) by PCR assay.
Results. Different patterns and frequency of virulence determinants were found for the *E. faecalis* and *E. faecium* isolates. *E. faecalis* isolates had more virulence determinants than *E. faecium* isolates. Clinical enterococcal isolates were found to possess more virulence determinants than *enterococci* isolated from faecal samples. The *esp* gene is significantly more common ($p = 0.049$) in vancomycin-resistant strains (85.7%) than in vancomycin-sensitive strains (44.2%). All of the vancomycin-resistant isolates were isolated from faecal samples. None of the classical virulence factors were found in 11% of enterococcal isolates, while all five virulence genes were found in 21% of enterococcal isolates.
Conclusions. All the virulence genes considered in this study were important in the pathogenesis of enterococcal infections and further studies including more virulence genes and epidemiological data will be necessary in order to analyze the association and role of virulence genes in the pathogencity of *enterococci* (**Adv Clin Exp Med 2015, 24, 1, 121–127**).

Key words: *Enterococcus,* virulent genes, *gelE*, *PAI*, *esp*, *ace*, and *sprE,* PCR.

Enterococci contribute to the normal flora of both the human and animal gastrointestinal tracts. In recent years, they have been reported to be a major cause of nosocomial infections [1], and they are among the most common pathogens isolated from infected surgical sites, septicemia and urinary tract infections. *Enterococcus faecalis* is responsible for about 80–90% of all enterococcal infections and *E. faecium* accounts for the remainder. An increasing number of strains resistant to large numbers of antimicrobial agents often cause fatal infections [2–3].

* This study was supported by Research University grant, USM RU grants 1001/PPSP/813020 and 1001/PPSP/813045 from University Sains Malaysia.

The genes responsible for vancomycin resistance in *enterococci* have been identified as *van*A, *van*B, *van*C, *van*D, *van*E, and *van*G based on gene structure and resistance characteristics. In addition, high-level gentamicin resistant (HLGR) strains have been encountered in large numbers of nosocomial enterococcal infections. The presence of virulent genes in enterococci seems to be associated with in-hospital spread and possibly with increased virulence [3]. Vancomycin-resistant *enterococci* in particular have emerged as a major cause of nosocomial infections [4]. A number of enterococcal virulence factors have been described to date, of which gelatinase (*gelE*), pathogenicity islands (*PAI*), enterococcal surface protein (*esp*), accessory colonization factor (*ace*) and serine protease (*sprE*) have been studied most intensively [2]. The extracellular zinc metalloprotease gelatinase, which hydrolyses gelatin and collagen, has been identified in dairy strains of *E. faecium* and has been shown to exacerbate endocarditis in an animal model [5–6]. Pathogenicity islands represent distinct genetic elements encoding virulence factors of pathogenic bacteria. They are a type of genomic islands, which are common genetic elements sharing a set of unifying features [7–8]. Enterococcal surface protein is important for the initial adherence and biofilm formation of *E. faecium* [9–10]. It has also been shown that *E. faecium* strains harboring the *esp* gene have significantly higher conjugation rates than strains without *esp* [11]. As Vankerckhoven et al. pointed out: "The enterococcal surface protein is chromosomally encoded and has an interesting structure that includes a central core consisting of distinct tandem repeat units. This central repeat region serves as a retractable arm, extending the N-terminal globular domain through the cell wall to the surface, which might facilitate immune evasion in case of immune deficiency. Enterococcal surface protein is associated with increased virulence, colonization and persistence in the urinary tract, and biofilm formation" [12]. A different *esp* gene has lately been seen in both vancomycin-resistant and susceptible *E. faecium* [3, 13]. The purpose of this study was to screen for the 5 potential virulence factors in *enterococci* and to investigate their prevalence in 3 Malaysian reference hospitals. Any possible correlation between the presence of potential virulence factors and the source of the enterococcal strain (human stool or clinical samples) was investigated.

Material and Methods

Bacterial Strains and Identification

All the laboratory procedures were performed at Microbiology Department Laboratories. Before commencing, the present study ethical approval was obtained from the University Sains Malaysia's Research and Ethics Committee. Two hundred twenty two enterococcal isolates (183 *E. faecalis* and 39 *E. faecium*) were obtained from clinical samples taken from patients between September 2001 and June 2004 within different wards of 3 referral hospitals in Malaysia – Hospital University Sains Malaysia (HUSM), General Hospital Kota Bharu (GHKB) and Hospital Kuantan (HKUN). Clinical specimens were isolated from blood, urine, exudates, sputum, stool and body fluid. All isolates were identified to the species level using Gram's stain; motility assessment; catalase production; growth in 6.5% NaCl; hydrolysis of L-pyrrolidonyl-b-naphthalamide; metabolism of xylose, mannitol, arabinose and sorbitol; bile hydrolysis and esculin pigment production; leucine aminopeptidase activity; and acidification of methyl-a-D-glucopyranoside. These tests were conducted as described by Turenne et al. [14].

Vancomycin MIC Test (E Test)

The MIC of vancomycin was determined using E test strips (AB Biodisk, Solna, Sweden), according to the manufacturer's instructions using MH agar with 2% NaCl.

Oligonucleotide Primers

The virulent genes of enterococci, *gel*E, *PAI*, *esp*, *ace*, and *spr*E were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). As the authors wrote in an earlier article for DNA sequence alignment and primer design, the ClustalW program in Vector NTI version 9.0 software (Invitrogen, Carlsbad, CA, USA) was used to align the DNA sequences. The conserved and non-conserved regions of the DNA sequence alignments were visualized using GeneDoc software [15]. The 5 oligonucleotide primer pairs (Research Biolabs, KL, Malaysia) used to amplify all virulent genes and the expected amplicon sizes are listed in Table 1. Primers for the detection of *gel*E (GenBank accession number M37185), *PAI* (GenBank accession number AF329367), *esp* (GenBank accession number DQ845099), *ace* (GenBank accession number AF260878), and *spr*E (GenBank accession number Z12296) were designed by our team from gene

Table 1. PCR primer sequences and products used for detection of virulence genes

Virulence factor	Gene	Primer name	Oligonucleotide sequence (5 , to 3')	Product size (bp)	Annealing temperature (TA)(°C)
Gelatinase	*gelE*	GEL-F GEL-R	CGA AGT TGG AAA AGG AGG C GGT GAA GAA GTT ACT CTG A	372	54
Pathogenicity island	*PAI*	PAI-F PAI-R	GAC GCT CCC TTC TTT TGA C CCA GAG AAA TTA CTA CCA T	387	54
Enterococcal surface protein	*esp*	ESP-F ESP-R	TTT GGG GCA ACT GGA ATA GT CCC AGC AAA TAG TCC ATC AT	407	56
Accessory colonization factor	*ace*	ACE-F ACE-R	CAG GCC AAC ATC AAG CAA CA GCT TGC CTC GCC TTC TAC AA	125	58
Serine protease	*sprE*	SPR-F SPR-R	GGT AAA CCA ACC AAG TGA ATC TTC TTC CGA TTG ACG CAA AA	300	56

bank database. The specificity of the primers was checked using BLAST, which is available at the GenBank website [16].

Bacterial Lysates for PCR

Bacterial lysates were prepared by suspending 1 mL overnight bacteria culture in LB broth with 200 μL DNase-free distilled water and boiling in a water bath for 10 min [15]. The bacterial lysates were used directly in PCR assays.

PCR for the Detection of Virulence Genes in Vancomycin-Resistant Enterococci

The PCR was performed using 2 μL of the bacterial lysates and primer pairs to amplify the target genes individually. PCR was performed in 20-μL reactions. Each reaction contained 1 pmol/μL of each forward and reverse primer, 160 uM dNTPs, 2.5 mM $MgCl_2$, 1 × PCR buffer, 0.75 U *Taq* DNA polymerase and 10 ng DNA template. PCR was performed using the Mastercycler 5330 (Eppendorf, Hamburg, Germany), with an initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at the TA of the primer pairs, and extension at 72°C for 30 s; followed by an extra cycle of annealing at the TA for 30 s and a final extension at 72°C for 5 min. The PCR products were electrophoresed through 2% low EEO agarose gel (Promega, Madison, USA) stained with ethidium bromide, at 100 volts for 60 min. PCR products were seen using an image analyzer.

Statistical Analysis

Chi-square analysis of contingency tables and Fisher's exact test were used for statistical analysis. IBM SPSS for Windows 19.0; (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. A p value < 0.05 was considered statistically significant.

Results

One hundred and ninety-eight (89%) of the tested enterococcal isolates possessed at least 1 of the 5 virulence factors, whereas in 24 (11%) of the isolates none of these classic factors was present. *E. faecalis* strains harbor significantly more virulence determinants than *E. faecium* strains, and a distinct trend was identified within the 5 genes Table 2. Forty-one (22.4%) *E. faecalis* isolates possessed all 5 virulence determinants compared with only five (12.8%) of the *E. faecium* isolates. All of the vancomycin-resistant isolates were isolated from faecal samples.

The prevalence of *esp* was significantly higher ($p < 0.001$) among clinical enterococcal isolates (81 of 137 [59.1%]) than among faecal isolates (21 of 85 [24.7%]). The *esp* gene was significantly more prevalent ($p = 0.05$) among the vancomycin-resistant enterococcal (VRE) isolates than among the vancomycin-sensitive isolates (VSE), with 6 of 7 (85.7%) VRE isolates vs 95 of 215 (44.2%) VSE isolates carrying the gene Table 3. A significant difference in the prevalence of *esp* ($p = 0.042$) was found among the 171 HUSM isolates (132 *E. faecalis* isolates and 39 *E. faecium* isolates): 65 of 132 (49%) *E. faecalis* isolates vs 12 of 39 (31%) *E. faecium* isolates possessed this gene. However, no significant

Table 2. Association of virulence genes with species identification; vancomycin susceptibility and types of specimens

		Number of isolates possessing at least one virulence gene (%)	Number of isolates without any virulence genes (%)	p value	Number of isolates possessing all five virulence genes (%)	Number of isolates possessing fewer than five virulence genes (%)	p value
Species identification	*E. faecalis*	170 (92.9)	13 (7.1)	0.001**	41 (22.4)	142 (77.6)	0.18*
	E. faecium	28 (71.8)	11 (28.2)		5 (12.8)	34 (87.2)	
Vancomycin susceptibility test	sensitive	192 (89.3)	23 (10.7)	0.56**	46 (21.4)	169 (78.6)	0.349*
	resistant	6 (85.7)	1 (14.3)		0 (0)	7 (100)	
Type of specimen	stool	64 (75.3)	21 (24.7)	< 0.001*	11 (12.9)	74 (87.1)	0.024**
	clinical	134 (97.8)	3 (2.2)		35 (25.5)	102 (74.5)	

* – Fisher's exact test.
** – Pearson χ^2 test.

Table 3. Percent prevalence of virulence determinants *esp, gelE*, *sprE, PAI,* and *ace* among *E. faecalis* and *E. faecium* isolates

		esp		*gelE*		*sprE*		*PAI*		*ace*	
		No. (%)	p value	No. (%)	p value	No. (%)	p value	No. (%)	p value	No. (%)	p value
Species	*E. faecalis*	90 (49.2)	0.036	140 (76.5)	0.12	139 (76)	0.23	73 (39.9)	0.048	168 (91.8)	< 0.001
	E. faecium	12 (30.8)		26 (66.7)		26 (66.7)		9 (23.1)		27 (69.2)	
Total		102		166		165		82		195	
Vancomycin	sensitive	95 (44.2)	0.049*	162 (75.3)	0.28*	161 (74.9)	0.38*	81 (37.7)	0.21*	191 (88.8)	0.04*
	resistance	6 (85.7)		4 (57.1)		4 (57.1)		1 (14.3)		4 (57.1)	
Total		102		166		165		82		195	
Specimen	stool	21 (24.7)	< 0.001	59 (69.4)	0.15	59 (69.4)	0.19	17 (20)	< 0.001	62 (72.9)	< 0.001
	clinical	81 (59.1)		107 (78.1)		106 (77.4)		65 (47.4)		133 (97.1)	
Total		102		166		165		82		195	
Source	HUSM	78 (76.5)	0.98	126 (75.9)	0.71	125 (75.8)	0.67	63 (77.8)	0.18	145 (74.4)	NA
	HKTN	16 (15.7)		26 (15.7)		26 (15.8)		9 (11.1)		33 (16.9)	
Total		94		152		151		72		178	
	GHKB	8 (7.8)		14 (8.4)		14 (8.5)		9 (11.1)		17 (8.7)	

** – Pearson χ^2 test.

difference in the frequency of the *esp* gene was observed between different hospitals. The *esp* gene was significantly more common (p = 0.036) among *E. faecalis* isolates (90 of 183 [49.2%]) than *E. faecium* isolates (12 of 39 [30.8%]). The distribution of all virulence genes among different hospitals in Malaysia showed no significant differences.

The *gelE* gene was found in 107 of 137 (78.1%) clinical enterococcal isolates, whereas it was found in 59 of 85 (69.4%) faecal isolates (p = 0.15). No significant differences in the prevalence of the *gelE* gene were seen between the *E. faecalis* and *E. faecium* isolates or between vancomycin-resistant and sensitive isolates.

The distribution of the *sprE* gene among different enterococcal species, susceptibility to vancomycin and specimens source showed no significant differences. In addition, pathogenicity islands were found to be significantly more common in *E. faecalis* than in *E. faecium* (p = 0.048), and they

were also found to be more predominant in clinical samples than stool samples ($p < 0.001$). The majority of strains (88%) possessed the *ace* determinant. The percentage prevalence of *gelE, sprE*, *esp,* and *PAI* genes among all isolates were 74.8%, 74.3%, 45.5%, and 30.5%, respectively.

Discussion

Enterococci are important nosocomial pathogens and are one of the major causes of infection within hospitals. The ability to acquire genes encoding antibiotic resistance combined with a natural resistance to various antimicrobial agents and to extreme environments (such as low pH, high salinity and high temperatures) makes these bacteria exceptional survivors [2]. Only 13 (7.1%) *E. faecalis* isolates had none of the virulence genes, which is in contrast to the results of a previous study in which more than 45% of endocarditis *E. faecalis* isolates were found not to possess these factors [17]. For this reason, it was concluded that other factors may also be important in the pathogenesis of enterococcal infections [17]. A wide variety of genes encoding virulence factors have been detected among our clinical *Enterococcus faecalis* strains, which was relevant to recent studies [18–19]. The *esp* gene was detected in 45.5% of all enterococcal isolates, in agreement with the findings of other studies [20–21], and the same result was shown by Vankerckhoven et al. "which identified the *esp* gene in about 80% of *E. faecium* strains" [12]. However, this is in contrast to the findings of Shankar et al. [22], who reported the absence of *esp* in *E. faecium*. It was recently shown that the enterococcal *esp* gene is part of a large (150 kb) *PAI* [23]. The Esp protein has an important role in pathogenicity, as it promotes the primary attachment to biotic and abiotic surfaces and is involved in evasion of the host immune system [24]. Our study found that the *esp* gene was significantly more common among clinical enterococcal isolates than among faecal isolates. Again, a previous study demonstrated the absence of this trait in dairy *E. faecium* [24]. This study showed results relevant to the present situation [19, 25], in that the *esp* gene is significantly more prevalent ($p = 0.049$) in VRE strains (85.7%) than in VSE strains (44.2%). Results of previous studies on the frequency of *esp* in VRE and VSE were inconsistent; Willems et al. [3] showed a higher prevalence of *esp* among VRE strains than among VSE strains, whilst other studies demonstrated the opposite [26–27]. Yet other studies found an equal distribution of the *esp* gene among VRE and VSE strains [13]. Gelatinase has been shown to contribute to the virulence of enterococci in an animal model [28], but little is known about its significance in the pathogenesis of human enterococcal infections. In our study we found 140 (76.5%) gelatinase-producing *E. faecalis* strains, but their clinical significance was unclear. Gelatinase was detected in 26 (66.7%) *E. faecium* isolates. A previous study by Coque et al. showed 54% of *E. faecalis* endocarditis isolates, 68% of other blood culture isolates and 27% of community-acquired faecal isolates were gelatinase producers [17]. However, a statistically significant difference was not observed. Furthermore, Park et al. reported: "that when a collection of *E. faecalis* strains from clinical isolates was screened for the presence of virulence factor genes, the *gelE* gene was found to be the most common factor. In several animal infection models, *E. faecalis* s*prE* was identified as a critical virulence factor, although the mechanisms underlying its virulence remain to be fully elucidated. *sprE*-producing *E. faecalis* was shown, in a mouse peritonitis model, to elicit increased mortality compared to the *sprE*-defective mutant. Similarly, *E. faecalis sprE* was determined to contribute in concert to pathogenesis in a rabbit endophthalmitis model" [29]. The *PAI* gene was found to be significantly much more common among clinical isolates than stool isolates. *PAIs* are present in the genomes of pathogenic organisms but absent from the genomes of nonpathogenic organisms of the same or closely related species. *PAIs* were first described as chromosomal DNA regions, but the increasing amount of sequence data from extrachromosomal elements supports the view that *PAIs* may also be part of plasmids or bacteriophage genomes [30]. An earlier study related the presence of a putative *PAI* with an epidemic [8], since 13 of the 14 clones analyzed from different hospital outbreaks contained this *PAI* [3].

The *ace* gene, which codes for Adhesion of collagen from *E. faecalis* with characteristics similar to a collagen-binding protein of *Staphylococcus aureus*, was found to be significantly more common ($p < 0.001$) among *E. faecalis* (91.8%) than *E. faecium* (69.2%) isolates. This was similar to an earlier study [31]. A previous study identified the regulator of *ace* and demonstrated for the first time that *ace* is a virulence factor for *E. faecalis* [32].

Finally, the current study revealed that *ace* was found in 87.8% of all enterococcal isolates and the majority of these cases were faecal samples. This was not surprising since *E. faecalis* is naturally present in the gastrointestinal tract and has to survive in an environment containing substances like bile [32].

In conclusion, our results confirm the predominance of various virulence genes in clinical *E. faecalis* isolates. Therefore, *E. faecium* strains isolated from both clinical and faecal specimens have lower

pathogenic potential than *E. faecalis* isolates. We found that the clinical isolates carried more virulence genes than faecal isolates. This study proved the existence of an additional virulence gene in addition to those analysed in the current study. A sensible correlation between the presence of *esp* and *ace* genes with vancomycin resistance was found.

It was concluded that all the virulence genes considered in this study are important in the pathogenesis of enterococcal infections. Further studies are necessary in order to analyse the role of these virulence genes in the pathogenicity of *enterococci*.

Acknowledgments. We are grateful to Institute for postgraduate studies, University Sains Malaysia for their Fellowship support, and three referral hospitals in Malaysia: Hospital University Sains Malaysia, General Hospital Kota Bharu and Hospital Kuantan, Malaysia for providing the clinical isolates.

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Confilct of interest: None declared

Received: 29.05.2013
Revised: 28.02.2014
Accepted: 12.01.2015