Frequency of Virulence-Associated Genes in *Enterococcus faecalis* Isolated in Kuwait Hospitals

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Key Words

Enterococci • Virulence genes • Hemolysins • Aggregation substance

Abstract

Objective: The objective of this study was to investigate the carriage of 6 virulence-associated genes in *Enterococcus faecalis* isolates obtained from patients in 8 hospitals in Kuwait.

Materials and Methods: In total, 466 *E. faecalis* isolates were obtained from 313 urine samples, 68 wound swabs, 36 blood samples, 25 rectal swabs, 12 high vaginal swabs and 12 miscellaneous sources. Genes for gelatinase (*gelE*), aggregation substance (*aggA*), hemolysin activation factor (*cylA*), enhanced expression of pheromone (*eep*), enterococcal surface protein (*esp*), and *E. faecalis* endocarditis antigen A (*efaA*) were detected in PCR assays. **Results:** Of 466 isolates, 423 (90.8%) were positive for 1 and up to 5 genes. However, none of the genes was detected in all of the isolates. The prevalence of the individual genes was *eep*: 31.9%; *esp*: 31.5%; *gelE*: 28.5%; *efaA*: 27.9%; *aggA*: 23.4%, and *cylA*: 18.5%. Of the 423 positive isolates, 148 (34.9%) were positive for 2 genes and 52 (12.3%), 15 (3.5%) and 5 (0.9%) isolates were positive for 3, 4 and 5 virulence genes, respectively. The *efaA* and *esp* combination was detected in isolates from all clinical sources.

Conclusion: The study showed a high prevalence of virulence genes in *E. faecalis* isolated in Kuwait hospitals. The absence of a dominant gene in all of the isolates suggests that infections by *E. faecalis* may require the involvement of multiple virulence factors.

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Introduction

Enterococci are ubiquitous Gram-positive cocci. Although initially considered as commensal organisms in humans, they are now recognized as important causes of health care-associated infections including urinary tract infections, postsurgical wound infections, bacteremia, endocarditis, meningitis, neonatal sepsis and infections in transplant patients with *Enterococcus faecalis* and *E. faecium* responsible for the majority of these infections [1–3]. This is attributable to their acquisition of various putative virulence determinants and multidrug resistance [4–7].

Studies using animal models have indicated that strains that are involved in disease causation possess putative virulence factors that are lacking in those that constitute members of the normal flora [4, 6, 7]. These studies suggest that hemolysins, enterococcal surface proteins, gelatinase, cytolysin, enterolysin A, aggregation substance protein, collagen-binding adhesin and endocarditis antigen are important contributors to the virulence of enterococci [3, 7–10].

Hemolysin is an extracellular toxin that causes lyses of human, horse and rabbit erythrocytes [3, 5]. It is also known as cytolysin, because it targets a broad range of cells including eukaryotic and prokaryotic cells [11, 12]. Cytolysin is carried on pathogenicity islands with operons that include 5 genes (*cylLL*, *cylLS*, *cylA*, *cylB* and *cylM*) which are responsible for cytolysin production and regulation of expression [3, 9]. Cytolysin-producing *E. faecalis* strains have been associated with increased se-

verity of human infections [11] and enhance the virulence of *E. faecalis* in murine peritonitis [5] and rabbit endophthalmitis [12]. Gelatinase, encoded by *gelE*, is an extracellular zinc endopeptidase that hydrolyses collagen, gelatin and small peptides, and gelatinase-producing strains of *E. faecalis* have been implicated in endocarditis using animal models [3].

Enterococcal surface protein (Esp) is a cell wall-associated protein in *E. faecalis*, and its gene (*esp*) has been found to be significantly higher among isolates recovered from infected patients than among other isolates [13]. Esp has been shown to enhance the persistence of *E. faecalis* in the urinary bladder in an experimental urinary tract infection [7]. The aggregation substance protein (As) is a pheromone-inducible protein of *E. faecalis* that promotes mating aggregates during bacterial conjugation. It is encoded by pheromone response plasmids and mediates efficient donor-to-recipient contact to facilitate plasmid transfer [14]. It mediates adherence to host cells, increased cell surface hydrophobicity and resistance to phagocytosis [3]. The production of normal levels of pheromones requires the presence of an intramembrane protein, the enhanced expression of pheromone (Eep) encoded by *eep* [15]. Eep is involved in the processing of pheromone precursor, the regulation of its expression or secretion. The *E. faecalis* endocarditis antigen (Efa A) is an adhesin with homology to cell surface proteins from other streptococci such as *Streptococcus parasanguis*, *S. gordonii* and *S. pneumoniae* [16].

Although we have previously studied Enterococcus species obtained from clinical sources in Kuwait hospitals for the prevalence of antibiotic resistance in Udo et al. [17, 18], there are no studies documenting the carriage of putative virulence determinants in *E. faecalis* obtained from patients in Kuwait hospitals and the Arabian Gulf region. Therefore, in this study, we investigated *E. faecalis* isolated from different clinical samples in Kuwait hospitals for the presence of genes for putative virulence factors to provide initial data on this important subject in the country.

Materials and Methods

E. faecalis Isolates

A total of 466 nonduplicate *E. faecalis* isolates were obtained from 313 urine samples, 68 wound swabs, 36 blood cultures, 12 high vaginal swabs (HVS), 25 rectal swabs, 5 catheter tips, 4 pleural fluids, and 1 each of bile, cerebrospinal fluid and semen samples in 8 hospitals in Kuwait between March 1, 2005 and June 30, 2007. The hospitals were: Maternity (106 isolates); Jahra (94 iso-

lates); Farwaniya (59 isolates); Mubarak (52 isolates); Al-Sabah (48 isolates); Amiri (29 isolates); Al-Razi (23 isolates), and Adan (16 isolates). The origins for 39 isolates were not provided. Mubarak and Amiri hospitals are tertiary care hospitals while the others are general hospitals. All clinical samples were grown on brain heart infusion agar plates or brain heart infusion broth and incubated at 37°C for 24 h. The isolates were identified by cultural characteristics, Gram stain, catalase, and bile solubility tests. Further identification was performed by biochemical tests using API Strep (BioMerieux, SA, France).

Bacterial DNA Extraction for PCR

DNA was extracted from *E. faecalis* following a protocol described previously [18]. Two or 3 single colonies of an overnight growth of enterococci were picked using tooth picks and inoculated into 500 µl of brain heart infusion broth and centrifuged at 13,000 rpm for 5 min. The resultant pellet was resuspended in 300 µl sterile distilled water and the suspension was heated at 100°C in a boiling water bath to lyse the cells. The cellular debris was removed by centrifugation at 13,000 rpm for 5 min. The template DNA contained in the supernatant was stored at -20°C until required for PCR amplification.

Amplification of Genes for Virulence Markers

PCR was used to detect the presence of each of 6 virulence genes using specific primers and conditions published previously [19]. All of the 466 *E. faecalis* isolates were investigated for the carriage of genes for aggregation substance (*aggA*), hemolysin activator (*cylA*), enhanced expression of pheromone (*eep*), enterococcal surface protein (*esp*), gelatinase (*gelE*) and *E. faecalis* endocarditis antigen A (*efaA*). Their nucleotide sequences and expected sizes of the amplified products are listed in table 1. PCR was performed in a final volume of 25 µl HotStarTaq Master mix (Qiagen) containing 1× PCR buffer, 2.5 units HotStarTaq DNA polymerase, 200 µM of each dNTP, 50 pmol of each forward and reverse primers and approximately 1 µl of DNA. The reaction mixture was amplified in an Eppendorf Mastercycler (Eppendorf, Germany) with an initial activation step of 15 min at 95°C, followed by 30 cycles at 94°C for 45 s, annealing at a temperature specific for each primer for 1 min, extension at 72°C for 1 min and a final extension step at 72°C for 10 min. A negative control containing all reagents except target DNA template was included with each batch of PCR. An internal control consisting of primers CO1 and CO2 for 16S rRNA gene was included used to confirm Enterococcus species. *E. faecalis* strain ATCC29212 was used as positive control for *gelE*, *esp*, and *cylA*. *E. faecalis* strain DS16 was used as positive control for *aggA*, and *E. faecalis* strain OGIX was used as positive control for *eep* [15]. Amplified products were detected by agarose gel electrophoresis using 2.0% agarose (w/v) in TAE buffer for 2 h at 70 V. *E. faecalis* strain OGIX was a gift from Dr. D.B. Clewell, University of Michigan, Ann Arbor, Mich., USA.

Results

The distribution of the genes for virulence markers are summarized in table 2. Of the 466 *E. faecalis* isolates, 423 (90.8%) isolates were positive for at least 1 of the 6 viru-

Table 1. Oligonucleotide primers for the amplification of virulence genes in *E. faecalis*

Virulence gene	Nucleotide sequence (5'-3')	Amplicon size, bp
<i>aggA</i>	TE3: AAGAAAAAGTAGACCAAC TE4: AACGGCAAGACAAGTAAATA	1,553
<i>cylA</i>	TE17: TGGATGATAGTGATAGGAAGT TE18: TCTACAGTAAATCTTTCGTCA	517
<i>efaA</i>	TE5: GACAGACCCTCACGAATA TE6: AGTTCATCATGCTGCTGTAGTA	705
<i>esp</i>	Esp11: TTGCTAATGCTAGTCCACGACC Esp12: GCGTCAACACTTGCATTGCCGAA	933
<i>gelE</i>	gelE1: GTACAGGCATCTTTGTTGGA gelE2: GATTCATTCAAGGACCTGA	1,004
<i>eep</i>	eepF1: GAGCGGGTATTTTAGTTCTGT eepR1: TACTCCAGCATTGGATGCT	937
16S rRNA	CO1: AGTTTGATCCTGGCTCAG CO2: TACCTTGTTACGACTT	1,500

agg = Aggregation substance; *cylA* = activation of hemolysin; *eep* = enhanced expression of pheromone; *efaA* = *E. faecalis* endocarditis antigen A; *esp* = enterococcal surface protein; *gelE* = gelatinase.

Table 2. Distribution of genes for virulence markers among *E. faecalis* isolates

Sources of enterococci	<i>E. faecalis</i> -harboring genes for virulence markers distributed according to clinical sources					
	<i>eep</i>	<i>esp</i>	<i>gelE</i>	<i>efaA</i>	<i>aggA</i>	<i>cylA</i>
Urine (n = 313)	95 (30.3)	95 (30.3)	84 (26.8)	67 (21.4)	73 (23.3)	47 (15.0)
Wound (n = 68)	24 (35.3)	18 (26.4)	18 (26.4)	24 (35.3)	13 (19.1)	20 (29.4)
Blood (n = 36)	12 (33.3)	10 (27.7)	15 (41.6)	14 (38.8)	13 (36.1)	6 (16.6)
HVS (n = 12)	6 (50.0)	6 (50.0)	3 (25.0)	2 (16.6)	1 (8.3)	1 (8.3)
Rectal swab (n = 25)	7 (28.0)	10 (40.0)	4 (16.0)	18 (72.0)	4 (16.0)	7 (28.0)
Others (n = 12) ¹	5 (41.6)	8 (66.6)	9 (75.0)	5 (41.6)	5 (41.6)	5 (41.6)
Total (n = 466)	149 (31.9)	147 (31.5)	133 (28.5)	130 (27.9)	109 (23.4)	86 (18.5)

Figures in parentheses indicate percentages.

¹ Others consisted of cerebrospinal fluid (n = 1), catheter tips (n = 5), pleural fluids (n = 4), bile (n = 1), and semen (n = 1).

lence-associated genes. Forty-three (9.2%) isolates, consisting of 38 from urine and the remaining 5 from wound samples, yielded negative results for all of the investigated genes.

The genes for enhanced expression of pheromone, *eep*, and for enterococcal surface protein, *esp*, were detected in 149 (31.9%) and 147 (31.5%) of the isolates, respectively, making them the most prevalent virulence-associated genes in these isolates. The other common virulence-as-

sociated genes were *gelE*: 133 (28.5%), *efaA*: 130 (27.9%), and *aggA*: 109 (23.4%). The gene for hemolysin activator, *cylA*, was detected in only 86 (18.5%) of the isolates. None of the virulence markers was detected in all isolates.

The virulence-associated genes were detected in isolates from all clinical sources, including 282 (90.1%) urine samples, 61 (89.7%) wound swabs, 36 (100%) blood samples, 9 (75.0%) HVS, 24 (96.0%) rectal swabs, and 12 (100%) miscellaneous sources. The results presented in

table 2 also show that the individual virulence-associated genes were fairly distributed in isolates from the different clinical sources except for *aggA* and *cylA* that were rare in isolates from HVS.

The majority of the isolates were positive for 2 or more virulence genes. The number of virulence marker ranged between 1 and 5 genes per isolate. Of the 423 positive isolates, 204 (48.2%) were positive for a single virulence gene, whereas 219 (51.6%) of them were positive for 2 or more virulence genes. The different combinations of genes detected among the *E. faecalis* isolates are shown in table 3. One hundred and forty-eight (34.9%) of the isolates yielded 2 virulence markers, 52 (12.3%), 15 (3.5%) and 4 (0.9%) of the isolates were positive for 3, 4 and 5 virulence markers, respectively. The *efaA* + *esp* combination was more common followed by *eep* + *efaA*, *eep* + *esp*, and *efaA* + *gelE* combinations. The *efaA* + *esp* combination was detected in isolates obtained from all clinical sources. The *eep* + *efaA* and *efaA* + *gelE* combinations were detected in isolates from all sources except HVS, whereas the rest were mostly distributed among urinary or wound isolates.

Discussion

This study sought to provide initial data on the distribution of virulence-associated genes in *E. faecalis* obtained from clinical samples in Kuwait hospitals. The results demonstrated the presence of all 6 virulence-associated genes in 90.8% of *E. faecalis* isolates. The 6 virulence genes were distributed in isolates obtained from all clinical samples, although *aggA* and *cylA* were rare in HVS isolates. Similarly, *E. faecalis* obtained from clinical [19, 20] and food [20] sources have been shown to contain virulence markers.

The gene for the expression of pheromone, *eep*, was the most prevalent virulence gene in this study. In contrast, *efaA* was the most prevalent virulence-associated gene in clinical *E. faecalis* isolated in Brazil [19] and Italy [9], whereas *aggA* was the most prevalent virulence gene detected in *E. faecalis* in Sweden [21]. These results suggest regional differences in the carriage of dominant virulence determinants by *E. faecalis* isolates. The low prevalence of the hemolysin activator gene, *cylA*, in this study has also been reported in studies conducted in Sweden [21] and the USA [22].

Our results that the *E. faecalis* isolates contained at least 1 and up to 5 virulence markers was similar to a report from a Brazilian hospital where *E. faecalis* isolates

harbored between 1 and 8 virulence-associated genes [19]. However, despite the detection of all 6 virulence markers investigated in this study, there was no dominance of any of the genes or gene combinations. Apart from the *efaA* + *esp*, *eep* + *efaA* and *eep* + *esp* combinations that were detected in 26, 19 and 19 isolates, the other gene combinations were found in fewer isolates. The absence of a single virulence-associated gene in all of the isolates suggests that the virulence capacity of *E. faecalis* may depend on multiple rather than on individual virulence factors [23]. The manifestation of *E. faecalis* infection may require the interplay of multiple genes that are responsible for the secretion and regulation of the expression of the virulence factors [24].

The prevalence of individual virulence-associated genes in this study was lower than that reported in other studies. None of them was detected in up to 50% of the *E. faecalis* isolates. The most common gene, *eep*, in this study was detected in 31.9% of the isolates which was lower than the 58.9 and 73% prevalence of *eep* detected in *E. faecalis* isolates from Brazil [19] and Sweden [21], respectively. The low prevalence of the virulence-associated genes in this study could be due to the clinical conditions of the patients. Some studies have shown that *esp* was significantly higher among isolates obtained from infected patients than among colonization isolates [6, 15, 25]. In addition, studies by Warr et al. [8] suggested that *aggA* may be associated with infection because it was isolated more frequently in *E. faecalis* from blood cultures of transplant patients. Furthermore, *aggA*, *cylA*, *esp* and *gelE* were more common in *E. faecalis* isolates obtained from patients in the intensive care unit than in isolates from individuals in the community [26]. Our isolates were obtained from a mixture of infection and colonization sources. This may explain the low prevalence of the individual virulence determinants in line with the findings that *E. faecalis* obtained from different sources possessed different patterns of virulence factors [13, 26]. Although 9.2% of the isolates yielded no positive results for any of the 6 genes tested, they may still harbor virulence determinants that were not investigated in this study. They may also contain genes that are yet to be described.

Conclusion

This study has demonstrated that *E. faecalis* isolates obtained from clinical samples in Kuwait hospitals harbor a rich variety of virulence-associated genes. Although

Table 3. Carriage of multiple genes for virulence markers in *E. faecalis* from different clinical sources

Virulence genes	Urine (n = 313)	Wound (n = 68)	Blood (n = 36)	HVS (n = 12)	Rectal swab (n = 25)	Others (n = 12)	Total (n = 466)
Two genes							
<i>efaA, esp</i>	15 (4.8)	3 (4.4)	2 (5.6)	1 (8.3)	3 (12.0)	2 (16.6)	26 (5.6)
<i>eep, efaA</i>	9 (2.8)	5 (7.3)	3 (8.3)	–	1 (4.0)	1 (8.3)	19 (4.1)
<i>eep, esp</i>	15 (4.8)	–	1 (2.8)	3 (25.0)	–	–	19 (4.1)
<i>efaA, gelE</i>	9 (2.8)	4 (5.9)	4 (11.1)	–	1 (4.0)	1 (8.3)	19 (4.0)
<i>eep, gelE</i>	9 (2.8)	–	1 (2.8)	1 (8.3)	–	4 (33.3)	15 (3.2)
<i>aggA, gelE</i>	8 (2.5)	2 (2.9)	2 (5.6)	–	–	–	12 (2.6)
<i>cylA, gelE</i>	5 (1.6)	2 (2.9)	2 (5.6)	–	1 (4.0)	–	10 (2.1)
<i>cylA, efaA</i>	1 (0.3)	5 (7.3)	–	–	1 (4.0)	1 (8.3)	8 (1.7)
<i>cylA, aggA</i>	6 (1.9)	2 (2.9)	–	–	–	–	8 (1.7)
<i>aggA, eep</i>	2 (0.6)	1 (1.4)	1 (2.8)	–	–	–	4 (0.8)
<i>cylA, eep</i>	3 (0.9)	–	–	–	–	–	3 (0.6)
<i>cylA, esp</i>	1 (0.3)	–	1 (2.8)	1 (8.3)	–	–	3 (0.6)
<i>esp, gelE</i>	2 (0.6)	–	–	–	–	–	2 (0.4)
Three genes							
<i>aggA, efaA, gelE</i>	3 (0.9)	3 (4.4)	1 (2.8)	–	1 (4.0)	–	8 (1.7)
<i>cylA, efaA, esp</i>	3 (0.9)	–	1 (2.8)	–	3 (12.0)	–	7 (1.5)
<i>eep, efaA, esp</i>	3 (0.9)	1 (1.4)	–	–	3 (12.0)	–	7 (1.5)
<i>aggA, eep, esp</i>	3 (0.9)	1 (1.4)	1 (2.8)	–	–	–	5 (1.1)
<i>aggA, cylA, gelE</i>	4 (1.2)	1 (1.4)	–	–	–	–	5 (1.1)
<i>aggA, eep, gelE</i>	3 (0.9)	1 (1.4)	–	–	–	–	4 (0.8)
<i>aggA, cylA, eep</i>	2 (0.6)	–	1 (2.8)	–	–	–	3 (0.6)
<i>cylA, efaA, gelE</i>	2 (0.6)	–	–	–	1 (12.0)	–	3 (0.6)
<i>eep, efaA, gelE</i>	1 (0.3)	–	1 (2.8)	1 (8.3)	–	–	3 (0.6)
<i>aggA, eep, efaA</i>	2 (0.6)	–	–	–	–	–	2 (0.4)
<i>aggA, esp, gelE</i>	2 (0.6)	–	–	–	–	–	2 (0.4)
<i>eep, esp, gelE</i>	2 (0.6)	–	–	–	–	–	2 (0.4)
<i>cylA, eep, gelE</i>	–	–	–	1 (8.3)	–	–	1 (0.1)
Four genes							
<i>aggA, eep, esp, gelE</i>	5 (1.6)	1 (1.4)	–	–	–	–	6 (1.2)
<i>aggA, cylA, eep, esp</i>	1 (0.3)	2 (2.9)	–	–	–	–	3 (0.6)
<i>aggA, cylA, eep, gelE</i>	1 (0.3)	1 (1.4)	–	–	–	–	2 (0.4)
<i>aggA, cylA, efaA, gelE</i>	1 (0.3)	–	–	–	–	–	1 (0.2)
<i>aggA, cylA, eep, efaA</i>	1 (0.3)	–	–	–	–	–	1 (0.2)
<i>cylA, eep, esp, gelE</i>	–	1 (1.4)	–	–	–	–	1 (0.2)
<i>cylA, eep, efaA, esp</i>	1 (0.3)	–	–	–	–	–	1 (0.2)
Five genes							
<i>aggA, cylA, eep, esp, gelE</i>	2 (0.6)	1 (1.4)	–	–	–	–	3 (0.6)
<i>aggA, cylA, eep, efaA, gelE</i>	1 (0.3)	–	–	–	–	–	1 (0.2)

agg = Aggregation substance; *cylA* = activation of haemolysin; *eep* = enhanced expression of pheromone; *efaA* = *E. faecalis* endocarditis antigen A; *esp* = enterococcal surface protein; *gelE* = gelatinase.

none of the genes was detected in all of the isolates, the carriage of multiple genes by the majority of the isolates highlights the importance of these genes to the bacteria. Nevertheless, this study has provided initial data on the distribution of virulence-associated genes in *E. faecalis* isolated in Kuwait and in the Arabian Gulf countries.

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