ORIGINAL ARTICLE

Molecular detection of virulence factors and antibiotic resistance pattern in clinical *Enterococcus faecalis* strains in Sardinia

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Key words

Enterococcus faecalis • Virulence factors • Antibiotic resistance

Summary

In this study, the antibiotic resistance pattern and the presence of genes encoding several virulence factors in 91 Enterococcus faecalis strains isolated from different human clinical sources in Sardinia were investigated. Genotypic determination of virulence genes (gelE, esp, agg, ace, cylA,B,M,L_L,L_S, efaA, fsrB) was carried out by PCR. The production of gelatinase and haemolytic activity were also determined. Antimicrobial susceptibility tests were performed by an automated microdilution test (Vitek). The strains examined in this study contained at least one and up to as many as all virulence genes investigated. Examining the distribution of these factors in the different groups of clinical strains, we found that all but one virulence determinant were detected more frequently among urinary isolates. The detection of some factors by PCR did not always correlate with its phenotypic expression. Antibiotic susceptibilities among the Enterococcus faecalis strains investigated in our study were typical for the species, with expected levels of acquired resistance. Faecal isolates had the highest percentage of resistance, especially to high level-gentamicin, ciprofloxacin and norfloxacin. In summary, a wide variety of genes encoding virulence factors have been detected among our clinical Enterococcus faecalis strains, and those isolated from UTI were characterized by a higher virulence potency compared with strains from other clinical sources. Silent virulence genes (cyl or gelE) were frequently detected, therefore both the genotypic and phenotypic assays seem necessary for a better characterization of the strains. Our results may serve as a basis for additional surveillance studies of infections caused by this microorganism.

Introduction

Enterococcus faecalis is a normal commensal in the human and animal gastrointestinal tract but it has became increasingly recognised as one of the leading cause of nosocomial infections. Urinary tract infections (UTI) are the most frequent, although more serious infections, such as bacteremia, endocarditis and neonatal infections also occur [1].

One of the main reasons why enterococci can survive in the hospital environment is their resistance to a variety of antimicrobials. In fact, in addition to their intrinsic resistance to low levels of aminoglycosides, cephalosporins, lincosamydes and many β-lactams, enterococci are also able to acquire resistance to many antibiotics, either by mutation of existing chromosomal genes or by transfer of resistance determinants [2]. The past two decades have therefore witnessed the rapid emergence of multidrug resistant enterococci [3].

In addition to antimicrobial resistance, several putative factors that may contribute to enhanced virulence have been described in *Enterococcus faecalis*, although the molecular mechanism of virulence is still not completely understood. Adherence to host cells is considered to be a crucial step in the establishment of many bacterial infections, and a number of adhesion factors have been identified so far, such as the aggregation substance (AS), the endocarditis-associated antigen (EfaA), the enterococcal surface adhesin (Ace), the enterococcal surface protein (Esp) [4].

Secreted molecules, such as cytolysin and gelatinase, have also been indicated as possible virulence factors [4]. Gelatinase is a Zinc-endopeptidase, co-transcribed with the serine protease and regulated by the quorum-sensing *fsr* locus that can hydrolyze gelatine, collagen, casein and other small bioactive peptides. It has been shown that *Enterococcus faecalis* strains that contain *fsr* knockouts have diminished virulence both in mammalian [5] and *Caenorhabditis elegans* model [6].

Cytolysin is a bacterial toxin with haemolytic and bacteriolytic activity. The *Enterococcus faecalis* cytolysin operon has been characterized and the regulation of cytolysin expression described [7, 8].

It has been hypothesized that the presence of specific genes associated with virulence or invasiveness might enhance the ability of nosocomial enterococci to colonise hospitalized patients, but conflicting observations have been reported.

The purposes of this study were to define the antibiotic resistance pattern and to investigate the presence of genes encoding several virulence factors in *Enterococcus faecalis* strains isolated from different human clinical sources in Sardinia. The possible correlations

between the determinants possessed by the strains and their source of isolation were also investigated.

Methods

Clinical isolates

The *Enterococcus faecalis* strains analysed in this study were isolated from 91 patients (one isolate from each patient) attended at the Binaghi Hospital of Cagliari (Italy) and had the following sources: 55 from urine (patients with UTI), 20 from respiratory tract (pharyngeal swabs, bronchoalveolar lavage) and 16 from faeces (colonized patients).
The isolates were identified to species level using an automated system (Vitek, bioMérieux, Rome, Italy) as recommended by the manufacturer. To confirm the phenotypic identification, a PCR was performed with primers specific for the *Enterococcus faecalis* species [9]. All strains were stored at -20°C in 20% glycerol-BHI broth.

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were performed by an automated microdilution test (Vitek, bioMérieux), according to the manufacturer's instructions. Antimicrobials tested are reported in Table I.
Interpretation of results was according to CLSI (Clinical and Laboratory Standards Institute, formerly National Committee for Clinical Laboratory Standards) guidelines [10]. Isolates showing intermediate levels of susceptibility were classified as resistant.

Detection of haemolytic and gelatinase activity

Haemolytic activity was determined on Columbia Blood agar (Microbiol, Cagliari, Italy) plates supplemented with 5% defibrinated horse blood after 48 h of incubation at 37°C in anaerobiosis. β-haemolysis was indicated by a clear zone of hydrolysis around the colonies.
Production of gelatinase was tested on Todd-Hewitt agar (Microbiol) containing 30 g gelatine (Sigma, Milan, Italy) per liter. Plates were incubated overnight at 37°C, then placed at 4°C for 6 h before examination for zones of turbidity around the colonies, indicating hydrolysis.

PCR detection of virulence genes

The following virulence genes: *gelE* (gelatinase), *esp* (enterococcal surface protein), *ace* (enterococcal surface adhesion), *agg* (aggregation substance), the cytolysin operon (*cylA,B,M,L_L,L_S*), *efa A* (*Enterococcus faecalis* antigen A) and *fsrB* for *fsr* locus were amplified by PCR using published specific primers and conditions (Tab. I). The strains with *gelE* genotype but not producing gelatinase activity were examined for the presence of a 23.9 kb deletion segment corresponding to the *fsr* gene cluster upstream of *gelE* by PCR using the primer set and amplification conditions described by Nakayama et al. [11].

Oligonucleotides were synthesized by a custom primer service (BSC Biotech, Sardinia, Italy). Template DNA was extracted using a synthetic resin (Instagene, Bio-Rad, Melville, NY) according to the manufacturer. Amplification reactions were performed in a Mastercycler gradient 5331 (Eppendorf).

Statistical analysis

Categoric analysis was done by the χ^2 method with Yates's correction, where appropriate, with significance limits posed at $P < 0.05$.

Results

Antimicrobial susceptibility tests

The comparative in vitro antimicrobial activities of the 13 agents investigated are shown in Table II. All strains, regardless of their origin, were susceptible to ampicillin, teicoplanin and nitrofurantoin. The majority were resistant to tetracycline and fosfomycin, while only three (one from each site of isolation) to vancomycin, although they were susceptible to teicoplanin.
Sensitivity rates to the other agents tested showed a certain variability according to the site of isolation. Faeces strains were considerably more resistant than those from other sources, showing elevated resistance rates to fluoroquinolones and high-level gentamycin ($P < 0.05$

Tab. I. PCR primers used for detection of virulence determinants in *Enterococcus faecalis* strains.

Gene	Primer	Sequence (5′ to 3′)	Product size (bp)	Reference
ace	F2	GAGCAAAAGTTCAATCGTTGAC	1,003	[31]
	R3	GTCTGTCTTTTCACTTGTTTCT		
agg	AGG1	AAGAAAAAGAAGTAGACCAAC	1,553	[19]
	AGG2	AAACGGCAAGACAAGTAAATA		
cylM	TE13	CTGATGGAAAGAAGATAGTAT	742	[19]
	TE14	TGAGTTGGTCTGATTACATTT		
cylB	TE15	ATTCCTACCTATGTTCTGTTA	843	[19]
	TE16	AATAAACTCTTCTTTTCCAAC		
cylA	TE17	TGGATGATAGTGATAGGAAGT	517	[19]
	TE18	TCTACAGTAAATCTTTCGTCA		
cylL_L	CYL_L1	GATGGAGGGTAAGAATTATGG	253	[8]
	CYL_L2	GCTTCACCTCACTAAGTTTTATAG		
cylL_S	CYL_S1	GAAGCACAGTGCTAAATAAGG	240	[8]
	CYL_S2	GTATAAGAGGGCTAGTTTCAC		
efaA	EFA1	GACAGACCCTCACGAATA	705	[19]
	EFA2	AGTTCATCATGCTGTAGTA		
fsrB	B1	ATGCTAATCGATTGGATTCTAAAA	710	[11]
	B2	TCTTTTTAGGTTTTTCAGTTTGTC		
gelE	GEL1	ACCCCGTATCATTGGTTT	419	[19]
	GEL2	ACGCATTGCTTTTCCATC		
esp	ESP11	TTGCTAATGCTAGTCCACGACC	933	[22]
	ESP12	GCGTCAACACTTGCATTGCCGAA		
ef1841/ fsrC	EF1841	GATCAAGAAGGGAAGCCACC	1050	[11]
	FSRC	CCAACCGTGCTCTTCTGGA		

Tab. II. Antimicrobial resistance in *Enterococcus faecalis* isolates according to the site of isolation.

Antibiotic	N (%) of resistant strains			
	Urine n = 55	Respiratory tract n = 20	Faeces n = 16	Total n = 91
Ampicillin	0	0	0	0
Penicillin G	5 (9)	0	2 (12.5)	7 (7.7)
Piperacillin	0	0	1 (6)	1 (1.1)
Imipenem	0	0	1 (6)	1 (1.1)
Gentamicin*	17 (31)**	8 (40)	12 (75)	37 (41)
Streptomycin*	23 (42)	6 (30)	4 (25)	33 (36)
Ciprofloxacin	17 (31)**	8 (40)	12 (75)	37 (41)
Norfloxacin	17 (31)**	8 (40)	12 (75)	37 (41)
Vancomycin	1 (1.8)	1 (5)	1 (6)	3 (3.2)
Teicoplanin	0	0	0	0
Tetracycline	48 (87)	17 (85)	16 (100)	81 (89)
Fosfomycin	52 (94.5)	20 (100)	15 (94)	87 (95.6)
Nitrofurantoin	0	0	0	0

* High-level

** $P < 0.05$ vs faeces

vs urine). Isolates from respiratory tract and urine were fully susceptible to piperacillin and imipenem, and showed a low degree of resistance to fluoroquinolones and high-level amynoglycosides. Only five urine strains were resistant to penicillin G.

Although 89% of isolates presented multidrug resistance, i.e. resistance to two or more drugs, only four isolates (three from faeces and one from urine) were resistant to all agents tested, the majority being resistant to two (30%) or five (19%). Interestingly, resistance to tetracycline and fosfomycin was always associated (17 strains from urine, 12 from faeces, 8 from respiratory tract) and all strains resistant to ciprofloxacin were also resistant to norfloxacin, suggesting the possibility of gene linkage, although this was not confirmed in our study.

PCR DETECTION OF VIRULENCE GENES

The results of PCR amplification of the putative virulence genes tested are reported in Table III. All genes were always detected in higher percentages in urine isolates, except for *esp*, which was recovered in higher but not significantly different percentage in strains from respiratory tract. A characteristic distribution in number of virulence factors could be noted. Isolates from UTI possessed between one (3.6%) and all seven (12.7%) determinants, with the majority (23.6%) having six, while strains from faeces and respiratory tract usually carried four factors (data not shown).

According to the type of factor, no distinct pattern could be observed but we found that 84% of positive *ace* strains were associated with *gelE* gene and 60% of positive *cylM* strains also possessed *agg* factor.

As for adhesins, *efaA* was detected in almost all isolates (96.7%), pointing to the widespread occurrence of this adhesion-associated factor in *Enterococcus faecalis*. The second most frequent adherence factor was *ace* (67%) followed by *esp* (52.7%), while *agg*, coding for the aggregation substance (AS), was detected in 42.8% of the strains.

After *efaA*, *gelE* represented the second most common factor, being present in 68% of total isolates. Of the determinants involved in the production of functional cytolysin, c*ylM*, whose product is involved in the post-translational modification of cytolysin, was the most commonly recovered (38.5% of total strains). As shown in Table IV, the whole operon $cylL_LL_SMBA^+$ was

Tab. III. Presence of putative virulence genes in *Enterococcus faecalis* strains according to the site of isolation.

Virulence factor	N (%) of isolates			
	Urine n = 55	Respiratory tract n = 20	Faeces n = 16	Total n = 91
efaA	55 (100)	19 (95)	14 (87.5)	88 (96.7)
ace	42 (76)	11 (55)	8 (50)	61 (67)
agg	28 (51)	7 (35)	4 (25)	39 (42.8)
esp	28 (51)	12 (60)	8 (50)	48 (52.7)
cylM	30 (54.5)	3 (15)	2 (12.5)	35 (38.5)
cylB	9 (16.4)	3 (15)	2 (12.5)	14 (15.4)
cylA	19 (34.5)	3 (15)	2 (12.5)	24 (26.4)
$cylL_L$	21 (38.2)	3 (15)	2 (12.5)	26 (28.6)
$cylL_S$	7 (12.7)	3 (15)	2 (12.5)	12 (13.2)
gelE	41 (74.5)	12 (60)	9 (56)	62 (68)
fsrB	30 (54.5)	10 (50)	6 (37.5)	46 (50.5)

Tab. IV. Correlation between the presence of the *cyl* operon genes and the detection of haemolysis in clinical strains of *Enterococcus faecalis* from different sources.

Source of isolates	Presence of genes of the *cyl* operon	Number of isolates	β-haemolysis positive*	β-haemolysis negative*
Urine (n = 55)	all set	7	7	-
	at least one *cyl*	26	20	6
	cyl not detected	22	-	22
Respiratory tract (n=20)	all set	3	3	-
	at least one *cyl*	0	-	-
	cyl not detected	17	-	17
Faeces (n=16)	all set	2	2	-
	at least one *cyl*	0	-	-
	cyl not detected	14	-	14

* number of isolates

detected in 12 isolates (seven from urine, three from respiratory tract and two from faeces). Genotypes with at least one gene were found in 26 urine isolates, while no *cyl* genes were detected in 53 strains (58%), with a significantly higher number of negative isolates among faeces and respiratory tract versus urine isolates (87.5, 85 and 40% respectively). Comparison of β-haemolysis and detection of *cyl* genes revealed that cytolysin determinants behave as silent in six non haemolytic urinary isolates.

Since the presence of *gelE* gene was also not always correlated with its expression, and *fsr* genes were shown to positively regulate the expression of gelatinase in *Enterococcus faecalis* [11, 12], we investigated the presence of a chromosomal deletion in the *fsr* gene cluster region upstream to *gelE*. As can be seen in Table V, of the 62 *gelE* positive strains 41, also possessing the *fsrB* gene, produced gelatinase. Among the 50 gelatinase negative strains, in 11 having *gelE* but lacking *fsrB*, the 1 kb PCR product corresponding to the 3' end of *ef1841* and 3' end of the *fsrC* was detected, indicating the presence of the 23.9 kb deletion sequence; 29 isolates lacked both *gelE* and *fsrB*, suggesting the presence of an even larger deletion and in five *fsrB*$^-$ *gelE*$^+$ strains the internal *ef1841/fsrC* product was not detected, presumably indicating a defective *fsr* gene cluster.

Discussion

In recent years, enterococci have received an increasing attention because of the development of resistance to multiple antimicrobial drugs and their common prevalence as nosocomial pathogens. In the case of *Enterococcus faecalis*, which is the third most common hospital pathogen, increased antibiotic resistance would likely result in increased mortality [13]. Antibiotic resistance may be considered to be both the cause and the effect of the adaptation of certain isolates to hospital environment, and the most likely antibiotics involved in this phenomenon are probably gentamicin and fluoroquinolones.

In our study, *Enterococcus faecalis* faecal isolates had the highest percentage of resistance, especially to high level-gentamicin, ciprofloxacin and norfloxacin. The percentage of faecal isolates showing HLR to gentamicin in this study is much higher (75%) than the value of 25% reported by d'Azevedo et al. [14] but similar to that found by Ruiz-Garbajosa et al. [15] among faecal ICU isolates (65.5%).

Although a major concern in our findings could be the presence of strains harbouring multiple antibiotic resistance, the elevated sensitivity rates to β-lactams and glycopeptides deserve particular attention. The low level of vancomycin resistance observed in our study (3% of total strains) confirms the only available data in literature for Sardinia [16], and is probably related to a rational use of vancomycin in the hospital from which the strains were isolated. Similar rates of glycopeptide resistance have been reported for *Enterococcus faecalis* strains isolated in Italy and Europe [17, 18].

Over the past few years, several studies have been focused on the putative virulence factors of enterococci of clinical, environmental and food origin. In general, the occurrence of virulence genes in *Enterococcus faecalis* strains varied widely from one study to another, probably due to the difference in their clinical and geographic origins. Most of these epidemiological studies have been carried out by PCR, which is widely used for rapid screening of the isolates.

The strains examined in this study contained at least one and up to as many as all virulence genes investigated. Examining the distribution of these factors in the different groups of clinical strains, we found that all but one

Tab. V. Phenotypes and genotypes related to ***fsr*** regulated gelatinase production in clinical isolates of ***Enterococcus faecalis*** from different sources.

	N (%) of isolates				
Source of isolates	Gelatinase Positive*	Gelatinase negative			
		fsrB$^-$ *gelE*$^-$	*fsrB*$^-$ *gelE*$^+$ *ef1841/fsrC*$^+$	*fsrB*$^-$ *gelE*$^+$ *ef1841/fsrC*$^-$	*fsrB*$^+$ *gelE*$^+$
Urine (n = 55)	30 (54.5)	14 (25)	9 (16)	2 (3.6)	0
Respiratory tract (n = 20)	9 (45)	8 (40)	1 (5)	1 (5)	1 (5)
Faeces (n = 16)	2 (12.5)	7 (43.7)	1 (6.2)	2 (12.5)	4 (25)
Total n (%) of isolates	41 (45)	29 (31.8)	11 (12)	5 (5.4)	5 (5.4)

* isolates having *fsrB*$^+$ *gelE*$^+$ genotype and gelatinase activity

virulence determinant, namely *esp*, were detected more frequently among urinary isolates. The high incidence of multiple virulence factors in urinary strains could potentially contribute to facilitate bacterial colonization and pathogenesis of *Enterococcus faecalis* in the urinary tract.

The very common occurrence of *efaA* is in agreement with previous findings [19, 20]. Surprisingly, in our study the occurrence of *esp* was not significantly associated with UTI isolates, although a role of Esp protein as urovirulence factor has been demonstrated [21]. However, the percentage of strains bearing *esp* was similar to that reported in other studies for *Enterococcus faecalis* [16, 18, 20]. Since the first studies on *esp* and its role in bacterial adhesion have been published [22, 23], several conflicting results have been reported. Tendolkar et al. [24] compared isogenic *esp*-positive and *esp*-deficient strains and found that *esp* leads to a significant increase in biofilm formation. However, Hällgren et al. [25] demonstrated that in vitro adherence to urinary tract catheters was indipendent by *esp*, and Kristich et al. [26] demonstrated that in vitro biofilm formation can occur in absence of *esp* while it is enhanced by GelE.

Among the *cyl* operon genes, *cylM* was present in 38.5% of total strains but was particularly associated with urinary isolates (56.5%), in accordance with the study of Creti et al. [20]. Reports on the incidence of these traits in *Enterococcus faecalis* have been contradictory; some indicate a low prevalence in isolates from food [27] or faeces of volunteers [28], whereas others have found a similar prevalence in both clinical and food strains [19]. To our knowledge this is the first study reporting the occurrence of the cytolysin operon genes in Italian clinical isolates.

The observed incidence of *agg* was lower than previously reported [19, 29]. Nevertheless, considering the existence of functional synergy between cytolysin and AS [30] and taking into account the physical clustering of *cyl* genes and adhesins genes, some supporting evidence for our findings can be found in the similar incidence of *agg* and *cyl* determinants in our strains.

As for the correlation between phenotypic and genotypic tests, in our study the detection of some factors by PCR did not always correlate with the phenotypic expression. We observed an 100% cytolysin phenotypic-genotypic congruence in strains carrying (12) or lacking (53) the whole operon, while in six phenotipically negative urinary isolates we found at least one of the genes investigated. In addition, 20 urinary strains produced β-haemolysis even if they did not possess the whole operon. Evidence for *cyl* gene variability due to genetic rearrangements was observed in other studies [8, 19]. Concerning gelatinase activity, the high frequency of apparently silent *gelE* genes among our isolates could be correlated with the loss of one or more genes of the *fsr* operon involved in the regulation of *gelE* expression, as reported by Nakayama et al. [11].

Conclusions

In summary, a wide variety of genes encoding virulence factors have been detected among our clinical *Enterococcus faecalis* strains, and those isolated from UTI were characterized by a higher virulence potency compared with strains from other clinical sources. Silent virulence genes (*cyl* or *gelE*) were frequently detected, therefore both the genotypic and phenotypic assays seem necessary for a better characterization of the strains. Our results may serve as a basis for additional surveillance studies of infections caused by this microorganism.

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