Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy)

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Enterococci are widely distributed in the environment; within the human body, they are normal commensals of the oral cavity, gastrointestinal tract and vagina. In recent years, enterococci have become one of the most frequent causes of acquired nosocomial infections worldwide. The molecular mechanism of virulence of these bacteria is still not completely understood. The aims of this work were to characterize phenotypically 47 isolates of *Enterococcus faecalis* and *Enterococcus faecium* collected in Sardinia (Italy) by their abilities to adhere to different epithelial cell lines (Vero and Caco-2 cells) and to associate their phenotypes with the presence of known virulence genes detected within their genomes by PCR. The following genes were amplified: AS (aggregation substance), *esp* (surface protein gene), *ace* (accessory colonization factor), *efaA* (*E. faecalis* endocarditis antigen) and *gelE* (gelatinase). The virulence genes were detected in *E. faecalis* isolates only, with the exception of *esp*, which was found in both species. The phenotypic and genotypic results were also compared with the susceptibility of isolates to various antibiotics.

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INTRODUCTION

Enterococci are a component of the normal intestinal flora. In recent years, they have been reported as a major cause of nosocomial infections (Jett *et al.*, 1994; Johnson, 1994), and they are among the most common pathogens isolated from infected surgical sites and blood-stream and urinary tract infections. *Enterococcus faecalis* is responsible for about 80–90% of all enterococcal infections and *Enterococcus faecium* accounts for most others (Jett *et al.*, 1994; Johnson, 1994). An increasing number of strains are resistant to large numbers of antimicrobial agents and often cause fatal infections (Jett *et al.*, 1994; Johnson, 1994; Willems *et al.*, 2001). In Italy, the significance of enterococci as a cause of nosocomial infections is increasing but, fortunately, vancomycin-resistant enterococci are not as common as in other countries of Europe and the USA (Baldassarri *et al.*, 2001; Sechi *et al.*, 1998a).

The interaction between enterococci and different epithelial cells has been analysed previously (Chow *et al.*, 1993; Kreft *et al.*, 1992; Schlievert *et al.*, 1998; Sußmuth *et al.*, 2000; Wells *et al.*, 2000). One of the aims of this work was to identify the role of the aggregation substance (AS) in the process of

adhesion of enterococci to renal and intestinal epithelial cells *in vitro* (Chow *et al.*, 1993; Kreft *et al.*, 1992; Sußmuth *et al.*, 2000). AS protein may be involved in virulence and is expressed on the surface of *E. faecalis*. The AS gene is present in pheromone-responsive plasmids (Sußmuth *et al.*, 2000; Wells *et al.*, 2000). AS facilitates aggregation of donor and recipient bacteria and helps in the transfer of conjugative plasmids (Chow *et al.*, 1993). The role of other genes was also investigated. Recently, various studies have reported the presence of the *esp* gene in isolates of *E. faecalis* and *E. faecium* (Satake *et al.*, 1997; Shankar *et al.*, 1999, 2001; Woodford *et al.*, 2001). Willems *et al.* (2001) reported a vancomycin-resistant *E. faecium* subpopulation, genetically distinct from non-epidemic vancomycin-resistant *E. faecium* (VREF) isolates, which was responsible of hospital epidemics in the USA, Australia and Europe. This subpopulation contained a variant of the *esp* gene that was absent in all non-epidemic and animal isolates. Identification of the variant *esp* gene may be important in guiding infection-control strategies. *Esp* possesses a structure with multiple repeat motifs, characteristic of many surface proteins involved in the process of adhesion to eukaryotic cells (Shankar *et al.*, 1999).

We analysed 47 *E. faecalis* and *E. faecium* isolates from clinical specimens such as urine and bronchoalveolar lavage (BAL) by detecting the virulence genes AS, *gelE*, *ace*, *efaA* and

Abbreviations: AS, aggregation substance; BAL, bronchoalveolar lavage.

esp by PCR and evaluating the ability of isolates to adhere to epithelial cell lines and to produce biofilms. The susceptibility of isolates to various antibiotics was also evaluated.

METHODS

Isolates. Forty-seven enterococcal isolates (15 *E. faecalis* and 32 *E. faecium*) were obtained from clinical samples taken from patients during 1998–2001 within different wards of the Hospital of Sassari (Sardinia, Italy). The 15 *E. faecalis* isolates were collected from vaginal swabs (*n* = 4), wound swabs (*n* = 3), BAL (*n* = 2), semen (*n* = 2), urinary devices (*n* = 2), pus (*n* = 1) and urine (*n* = 1). The 32 *E. faecium* isolates were collected from BAL (*n* = 17), pus (*n* = 4), perineum (*n* = 3), urine (*n* = 3), wound swabs (*n* = 2), catheter devices (*n* = 2) and heart devices (*n* = 1). All isolates were identified by the API ID32-Strep system (bioMérieux.)

DNA extraction and molecular typing. DNA was extracted as described previously (Sechi *et al.*, 1998a, b). Molecular typing was performed as described previously (Sechi *et al.*, 1998a).

Antibiotic susceptibility testing. Antibiotic susceptibility was assessed by using broth microdilution susceptibility tests to determine the MIC. Tests were performed in ready-to-use microtitre plates (Microdilution trays; Becton Dickinson) containing lyophilized antibiotics according to the manufacturer’s instructions. The MICs and levels of resistance were determined according to the recommendations of the NCCLS (Dicuonzo *et al.*, 2001). The antibiotics tested were penicillin, ampicillin, vancomycin, erythromycin, high-level gentamicin, teicoplanin, tetracycline, chloramphenicol and norfloxacin.

PCR for detection of *vanA* and *vanB*. PCRs were performed in a GeneAmp 2400 System thermal cycler (Applied Biosystems). The oligonucleotide primers for PCR amplifications were synthesized by Amersham Pharmacia Biotech. Primer sequences for *vanA* and *vanB* were those described by Satake *et al.* (1997). The reactions were performed in a total volume of 50 µl, using 5 µl crude cell lysate, 40 pmol of each of the four primers, 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each dNTP and 1.5 µl AccuPrime *Taq* DNA polymerase (Invitrogen Life Technologies). Amplification condi-

tions were as follows: an initial denaturation step of 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min, followed by a final elongation at 72 °C for 10 min. The PCR products were analysed on 1.2 % agarose gels stained with ethidium bromide solution and visualized under UV light. The clinical isolates *E. faecalis* A256 and *E. faecalis* Mn1, respectively used as positive controls for *vanA* and *vanB* resistance, were kindly provided by P. S. Cocconcelli (Università Cattolica del Sacro Cuore, Piacenza, Italy).

Detection of virulence genes by PCR. Primers and PCR conditions are given in Table 1. With the exception of primers for *esp* (Shankar *et al.*, 1999), primers were designed in this study.

Restriction digestion of *esp* amplified products. Amplified products from *esp* were digested with *Hind*III according to the supplier’s instructions (New England Biolabs).

Adhesion experiments. Caco-2 and Vero cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultivated in 24-well plastic dishes. Briefly, Caco-2 cells and Vero cells were cultivated in Dulbecco’s modified Eagle’s medium supplemented with 15 % fetal bovine serum and 4 mM L-glutamine. All tissue-culture reagents were obtained from Sigma. Cells were seeded at 2 × 10⁴ (Caco-2) or 2 × 10³ (Vero) cells per well and incubated at 37 °C in 9.5 % CO₂. Cells were used when they were semiconfluent. Cultures of *E. faecalis* and *E. faecium* were grown overnight in brain heart infusion (BHI) broth and diluted in the medium used for incubation of the cell lines. One millilitre, containing 10⁶ bacteria, was added to each well containing Caco-2 or Vero cells. Adhesion was estimated by counting the number of adhered bacteria per cell after incubation for 2 h at 37 °C and staining with May–Grunwald–Giemsa stain as described previously (Zanetti *et al.*, 1992).

Biofilm assay. The ability of the enterococcal isolates to form a biofilm on an abiotic surface was quantified as described by Toledo-Arana *et al.* (2001). Isolates were grown overnight at 37 °C in BHI broth plus 0.25 % glucose. The culture was diluted 1 : 20 in fresh BHI broth plus 0.25 % glucose at 37 °C and 200 µl of this suspension was used to inoculate sterile 96-well polystyrene microtitre plates (Iwaki). After 24 h at 37 °C, wells were washed with PBS, dried in an inverted position and stained

Table 1. PCR primers and reaction conditions

All PCR programs consisted of 30 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at the temperature shown and elongation at 72 °C for 1 min.

Target	Primer	Sequence (5'–3')	Product length (bp)	Annealing temperature (°C)
ace	ACE1	AAAGTAGAATTAGATCCACAC	320	56
	ACE2	TCTATCACATTCGGTTGCG		
gelE	gelE1	AGTTCATGTCTATTTCTTCAC	402	56
	gelE2	CTTCATTATTTACACGTTTG		
efaA	efaA1	CGTGAGAAAGAAATGGAGGA	499	56
	efaA2	CTACTAACACGTCACGAATG		
AS	AS1	CCAGTAATCAGTCCAGAAACAACC	406	54
	AS2	TAGCTTTTTTCATTCTTGTGTTTGTT		
esp	esp46	TTACCAAGATGGTTCTGTAGGCAC	A repeats*	58
	esp47	CCAAGTATACTTAGCATCTTTTGG		

*Primers esp46/esp47 (Shankar *et al.*, 1999) are specific for amplification of the A repeats.

with 1 % crystal violet for 15 min. The wells were rinsed once more and the crystal violet was solubilized in 200 µl ethanol/acetone (80 : 20, v/v). The A_{595} was determined using a microplate reader (Multiskan EC; LabSystems). Biofilm formation was scored as follows: –, non-biofilm-forming ($A_{595} \leq 1$); +, weak ($1 < A_{595} \leq 2$); ++, moderate ($2 < A_{595} \leq 3$); +++, strong ($A_{595} > 3$). Each assay was performed in triplicate and repeated three times.

RESULTS

The isolates of *E. faecium* and *E. faecalis* were evaluated for their antibiotic susceptibility (Table 2). All the *E. faecium* isolates exhibited resistance to a range of antibiotic substances. The highest percentages of resistant isolates were observed in the presence of penicillin, erythromycin and ampicillin (Table 2). A large number of isolates exhibited resistance to high levels of aminoglycosides, which predicts a lack of synergy between cell-wall-active agents (ampicillin, penicillin and vancomycin) and aminoglycosides. Only three clinical isolates (10 %) were resistant to vancomycin, with MICs greater than 32 µg ml⁻¹. All the other isolates were susceptible. Vancomycin and teicoplanin seemed to be the most effective antimicrobials, with about 90 % of isolates susceptible.

A large proportion of the *E. faecalis* isolates were resistant to tetracycline (73 %), as well as to penicillin (60 %) (Table 2). More than 30 % of the isolates were resistant to chloramphenicol. All *E. faecalis* isolates were susceptible or showed intermediate susceptibility to ampicillin, vancomycin and teicoplanin (Table 2).

To determine whether the isolates were clonal, all isolates were typed by ribotyping, as described previously (Sechi *et al.*, 1998a). Among the 32 *E. faecium* isolates analysed, 26 different ribotyping patterns were identified, whereas the 15 *E. faecalis* isolates generated 14 different ribotyping patterns (data not shown).

All isolates were screened for the presence of virulence genes identified previously in *E. faecalis* and *E. faecium* using primers designed in-house or published previously (Table 1). Nine *E. faecalis* isolates (60 %) were positive for *ace*, 13 (86.6 %) for *efaA*, 11 (73.3 %) for *gelE*, 5 (33.3 %) for AS and 9 (60 %) for *esp* (Table 3). Of the 32 *E. faecium* isolates, none yielded a product with the *ace*, *efaA*, AS or *gelE* primers. However, 23 isolates (71.9 %) generated the expected product when *esp* primers were used (Table 3).

When the *E. faecalis* isolates were analysed, primers targeting *ace*, *efaA*, *gelE* and AS generated bands of the expected sizes, whereas the *esp* primers generated bands of different sizes, as reported previously (Shankar *et al.*, 1999), although not all of them were multiples of 252 bp (Shankar *et al.*, 1999). On the basis of the numbers of bands and their molecular sizes, three patterns were identified for *E. faecalis* isolates and four patterns were identified for the *E. faecium* isolates (Fig. 1a). Amplification of *esp* from *E. faecalis* produced smaller bands in comparison with the bands generated from *E. faecium* (Fig. 1a). In order to verify that the variation in size was due to the presence of different numbers of A repeats, the

amplified bands were digested with *Hind*III (which cuts once within each A repeat unit) and the digestion products were visualized after electrophoresis on a 2 % agarose gel (Fig. 1b). In all cases, three major bands (of 173, 252 and 261 bp) of the expected size were obtained (Fig. 1b), as reported previously (Wells *et al.*, 2000). The number of A repeats varied from two (*E. faecalis* isolates) to six among most of the *E. faecium* isolates (Fig. 1a).

All 47 isolates were tested for their ability to adhere to renal (Vero) and intestinal (Caco-2) epithelial cells (Table 3). Both *E. faecalis* and *E. faecium* isolates adhered to Vero cells; however, some *E. faecalis* isolates (e.g. isolate 2) were highly adhesive, with more than 30 bacteria per cell (after examining more than 40 fields per sample containing at least 20 epithelial cells, each sample was done in triplicate). All *E. faecalis* isolates were more adhesive to Vero cells than to Caco-2 cells.

Various *E. faecium* isolates were adhesive to Vero cells, whereas only two isolates were found on Caco-2 cells (Table 3). To investigate a possible correlation between the presence of the *esp* gene and the capacity to adhere to epithelial cells and/or the capacity of the isolates to form biofilms, the capacity of the 47 enterococcal isolates to form biofilms on polystyrene microtitre plates was evaluated. The capacity for biofilm formation was scored as non-biofilm-forming, weak, moderate or strong as described in Methods (Satake *et al.*, 1997). In contrast to a previous study (Eaton & Gasson, 2001), the presence of the *esp* gene was not clearly associated with the capacity for biofilm formation (Table 3).

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 (Jett *et al.*, 1994; Johnson, 1994). Many of the *E. faecium* isolates tested were resistant to a wide range of antibiotics, such as penicillin, ampicillin and erythromycin; they were also resistant to aminoglycosides at a high level, from which one can predict that there is resistance to synergism between cell-wall-active agents (ampicillin, penicillin, vancomycin) and aminoglycosides. This is a serious problem, as it reduces the number of possible treatments available for enterococcal infections. A combination of penicillin or ampicillin plus an aminoglycoside is usually indicated for serious enterococcal infections, such as endocarditis. The high percentage (75 %) of clinical isolates resistant to ampicillin found in this study is alarming, as ampicillin is often the preferred treatment for enterococcal infections. Only three *E. faecium* clinical isolates (10 %) were resistant to vancomycin, with MICs greater than 32 µg ml⁻¹. All the other isolates were susceptible. Moreover, the three vancomycin-resistant isolates were also multidrug-resistant, and this is of concern, as this resistance may be transferred to

Table 2. Antibiotic resistance of *E. faecalis* and *E. faecium* isolates

Results were interpreted on the basis of MICs for enterococci according to Dicuonzo *et al.* (2001) and are scored as: S, susceptible; I, intermediate; R, resistant. VAN, Vancomycin; TEC, teicoplanin; AMP, ampicillin; PEN, penicillin; EM, erythromycin; TC, tetracycline; CL, chloramphenicol; NX, norfloxacin; GM, gentamicin.

Isolate	VAN	TEC	AMP	PEN	EM	TC	CL	NX	GM
<i>E. faecalis</i>									
1	I	I	S	R	I	R	I	S	S
2	S	S	S	I	R	R	R	S	S
3	S	S	S	I	R	R	R	S	R
4	S	S	S	I	R	R	R	S	R
5	I	S	S	R	I	R	S	S	S
6	S	S	S	I	I	R	R	S	R
7	S	S	S	R	I	R	S	R	R
8	S	S	S	S	I	S	S	I	S
9	S	S	S	R	I	R	S	S	S
10	I	S	S	R	I	R	S	S	S
11	S	S	S	R	I	S	I	I	S
12	I	S	S	R	I	S	S	I	S
13	S	S	S	I	I	I	S	S	S
14	S	I	S	R	I	R	S	R	R
15	I	S	S	R	R	R	R	S	S
<i>E. faecium</i>									
1	R	R	S	R	R	R	I	R	R
2	R	R	R	R	R	R	R	R	R
3	R	R	S	S	S	S	S	R	S
4	S	S	R	R	R	S	S	R	R
5	S	S	R	R	R	S	S	R	R
6	S	I	S	R	R	R	S	R	R
7	S	S	R	R	R	R	S	S	S
8	S	S	R	I	I	I	I	I	I
9	S	S	S	R	S	S	S	R	S
10	S	S	R	R	R	S	S	I	S
11	S	S	R	R	R	S	I	I	R
12	S	S	R	R	R	S	I	I	S
13	S	S	R	R	R	S	R	I	S
14	S	S	R	R	R	I	S	R	R
15	S	S	R	R	R	R	I	I	S
16	S	S	R	R	R	R	S	I	S
17	S	I	R	R	R	S	I	R	R
18	S	S	S	R	I	R	I	I	S
19	S	S	R	R	R	S	S	R	S
20	S	S	S	S	R	S	I	S	S
21	S	S	R	R	R	S	S	R	S
106	S	S	R	R	R	R	S	R	S
109	S	S	S	R	R	R	I	R	S
111	S	I	R	R	R	S	I	I	I
113	S	I	R	R	R	S	S	I	I
114	S	S	R	R	R	S	I	R	S
116	S	I	R	R	R	S	R	I	R
118	S	I	R	R	R	R	S	R	R
119	S	S	S	R	R	S	R	I	S
120	S	S	R	R	R	R	R	S	S
121	S	S	S	S	R	S	S	S	S
122	S	I	R	R	R	R	R	I	S

Table 3. Distribution of phenotypic and genotypic virulence determinants among *E. faecalis* and *E. faecium* isolates

Adhesion is scored as: –, non-adherent; +, weak (< 1 bacterium per cell); ++, moderate (~5 bacteria per cell); +++, strong (>10 bacteria per cell). Biofilm formation is scored as absent (–), weak (+), moderate (++) or strong (+++) as described in Methods.

Isolate	Source	Detection by PCR					Adhesion		Biofilm production
		<i>ace</i>	<i>efaA</i>	<i>gelE</i>	AS	<i>esp</i>	Vero	Caco-2	
<i>E. faecalis</i>									
1	Wound swab	+	+	+	—	+	—	—	++
2	Vaginal swab	+	+	—	+	+	+++	—	++
3	Urine	—	+	—	+	+	—	—	+++
4	Urinary device	—	+	+	—	+	—	—	++
5	Nail swab	+	+	+	—	—	+++	+	+
6	Wound swab	—	+	+	—	+	—	—	—
7	Pus	+	—	+	+	+	+	+	+
8	Sputum	—	—	+	—	—	—	—	+
9	Urinary device	+	+	+	—	—	+	—	+
10	Vaginal swab	+	+	—	—	—	++	+	++
11	Semen	+	+	+	—	+	++	+	+
12	Vaginal swab	+	+	+	—	—	+	—	+
13	Semen	—	+	—	+	+	+++	+	++
14	BAL	—	+	+	—	—	+	—	—
15	Vaginal swab	+	+	+	+	+	++	+	++
Positive [<i>n</i> (%)]		9 (60)	13 (86.6)	11 (73.3)	5 (33.3)	9 (60)			
<i>E. faecium</i>									
1	BAL	—	—	—	—	+	—	—	—
2	BAL	—	—	—	—	+	—	—	—
3	BAL	—	—	—	—	—	—	—	—
4	Pus	—	—	—	—	+	—	—	—
5	Perineum	—	—	—	—	—	—	—	—
6	BAL	—	—	—	—	—	—	—	—
7	BAL	—	—	—	—	+	—	+	—
8	BAL	—	—	—	—	+	—	—	—
9	BAL	—	—	—	—	+	—	—	—
10	BAL	—	—	—	—	+	—	—	—
11	BAL	—	—	—	—	+	—	—	—
12	BAL	—	—	—	—	+	—	—	—
13	BAL	—	—	—	—	+	—	—	+
14	BAL	—	—	—	—	+	—	—	—
15	BAL	—	—	—	—	+	—	—	+
16	Pus	—	—	—	—	+	—	—	—
17	Heart device	—	—	—	—	+	—	—	—
18	Pus	—	—	—	—	—	—	—	—
19	Wound swab	—	—	—	—	+	—	—	+
20	Perineum	—	—	—	—	+	—	—	+
21	Perineum	—	—	—	—	+	—	—	—
106	BAL	—	—	—	—	—	+	+	+
109	Pus	—	—	—	—	—	—	—	—
111	Urine	—	—	—	—	+	+	—	—
113	Urine	—	—	—	—	+	+	—	—
114	Urinary device	—	—	—	—	+	+	—	—
116	Urinary device	—	—	—	—	+	—	—	—
118	BAL	—	—	—	—	+	—	—	—
119	Wound swab	—	—	—	—	—	—	—	—
120	BAL	—	—	—	—	—	—	—	—
121	BAL	—	—	—	—	—	+	+	—
122	Urine	—	—	—	—	+	+	—	—
Positive [<i>n</i> (%)]		0	0	0	0	23 (71.9)

Fig. 1. Agarose gel electrophoresis of *esp* amplification products from *E. faecalis* and *E. faecium* isolates (a) and *Hind*III-digests of the *esp* PCR products (b). (a) Lanes: M, 100 bp ladder; 1–4, *E. faecalis* isolates 1 (lane 1), 3 (2), 11 (3) and 13 (4); 5–10, *E. faecium* isolates 1 (5), 8 (6), 10 (7), 14 (8), 106 (9) and 114 (10). (b) Lanes: M, 100 bp ladder; 1–4, *E. faecalis* isolates 1 (lane 1), 11 (2), 3 (3) and 13 (4); 5–8, *E. faecium* isolates 1 (5), 8 (6), 10 (7) and 14 (8).

more pathogenic micro-organisms. They were also resistant to high levels of aminoglycosides, with only one of them susceptible to penicillin. This means that the possible antimicrobial therapies for infections associated with such enterococcal strains are limited. Nonetheless, it is worth noting that the percentage of vancomycin-resistant enterococci isolated in the present investigation from human samples collected in Sardinian hospitals was very low, when compared with data reported from other countries.

Only three isolates from clinical samples gave the expected amplification product of 1030 bp when amplified with primers specific for *vanA*. These were the same isolates that were resistant to vancomycin and teicoplanin in antibiotic-susceptibility tests. No *vanB* genes were detected. The good fit between the phenotypic and genetic tests indicates that all the *vanA* genes present in the genomes of these isolates are actually expressed as acquired vancomycin resistance.

The 15 *E. faecalis* isolates showed elevated resistance to penicillin but not ampicillin, erythromycin or tetracycline; a small number of isolates were also resistant to gentamicin. None of the isolates was resistant to vancomycin or teicoplanin.

Enterococci are opportunistic pathogens and the immune system of the host is an important factor in the development of disease. Little is known about the mechanism of virulence of these bacteria and genes involved in the pathogenesis of disease have only recently been identified. *E. faecalis* is responsible for 80–90 % of all infections due to enterococci, and *E. faecium* is responsible for most of the remainder. *E. faecalis* and *E. faecium* show great differences in the incidence of virulence determinants (Toledo-Arana *et al.*, 2001; Zanetti *et al.*, 1992). On the basis of sequences of virulence genes available in the literature (Coque *et al.*, 1995; Eaton & Gasson, 2001; Ike *et al.*, 1987; Lowe *et al.*, 1995; Nallapareddy *et al.*, 2000; Schlievert *et al.*, 1998; Shankar *et al.*, 1999; Singh *et al.*, 1998; Su *et al.*, 1991), 47 clinical enterococcal isolates from patients of the Sassari Hospital in Sardinia (Italy) (15 *E. faecalis* and 32 *E. faecium*) were characterized genotypically, and their adhesion properties on Vero and Caco-2 epithelial cells and their capacity to produce biofilms were tested. Ribotyping confirmed that the isolates were not clonal.

The virulence determinants were present in different proportions of isolates of the two species. The *E. faecalis* isolates tested carried multiple virulence genes, whereas the *E. faecium* isolates tested were devoid of these virulence genes, except for *esp*. The results obtained for *esp* are different from the first report of Shankar *et al.* (1999). We found 71.9 % of *E. faecium* isolates positive for *esp*, whereas only 60 % of *E. faecalis* isolates yielded a PCR product. Other authors (Eaton & Gasson, 2001; Willems *et al.*, 2001; Woodford *et al.*, 2001) have reported similar prevalence of *esp* in *E. faecium* isolates from medical environments; *esp* was detected in 63–78 % of clinical isolates in the UK (Willems *et al.*, 2001; Woodford *et al.*, 2001). The large number of clinical *E. faecium* isolates positive for *esp* suggests a role in pathogenicity. Moreover, Shankar *et al.* (2001) suggest that Esp contributes to colonization and persistence of *E. faecalis* within the urinary tract. The *esp* primers used in this study amplify the A repeated region of the *esp* gene; our results show that larger numbers of A repeats were present within the *E. faecium* isolates tested (four different patterns generated) in comparison with three patterns found in *E. faecalis* isolates. To verify that variation occurred in the number of A repeats, the amplification products were digested with *Hind*III (which cuts once within each A repeat). The digestion confirmed our hypothesis; a similar observation has been reported for *E. faecalis* (Shankar *et al.*, 1999), where addition or deletion of *esp* repeat units led to changes in the size of the encoded protein. It was thought that Esp size variation at the cell surface could define an environment-specific function for Esp. Our results indicate no correlation between the epithelial cell-adhesion phenotype and the presence of *esp* for either *E. faecalis* or *E. faecium*.

Toledo-Arana *et al.* (2001) reported high sequence similarity between Esp and Bap (a biofilm-associated protein of *Staphylococcus aureus*); for this reason, the association of *esp* with the capacity to form biofilms on a polystyrene surface was investigated. A strong association between the presence of *esp* and biofilm formation in *E. faecalis* has been

reported (Toledo-Arana *et al.*, 2001). In the same study, it was suggested that the presence of *esp* rather than the phenotype (adherence or biofilm formation) is a good marker for identification of strains that are highly adherent to abiotic surfaces. Our results confirm this association for *E. faecalis* but not for the *E. faecium* isolates tested. For the *E. faecium* isolates, an association was found between the presence of *esp* and the capacity to grow in the presence of ampicillin. Of the nine *esp*-negative *E. faecium* isolates, six were susceptible to ampicillin, whereas, of the 23 *esp*-positive isolates, only three were susceptible to ampicillin.

The *ace* gene, previously reported to mediate adherence to collagen types I and IV and laminin (Nallapareddy *et al.*, 2000), was detected in nine of 15 *E. faecalis* isolates, but no amplification was obtained from the *E. faecium* isolates studied.

Enterococcus species have an efficient gene-transfer mechanism, including conjugation via the sex-pheromone plasmids and conjugative transposon. The chance that virulence factors may be acquired by conjugation is very high, as reported previously (Chow *et al.*, 1993; Coque *et al.*, 1995; Heaton *et al.*, 1996; Jett *et al.*, 1994). The adhesion experiments clearly confirmed that *E. faecalis* is more virulent than *E. faecium*, but none of the genes tested was always associated with the virulence phenotype. In particular, a large number of *E. faecalis* isolates were adhesive on Vero cells, whereas adhesion on Caco-2 cells was less evident and was observed in only few isolates. Bacterial adherence was not associated significantly with any of the virulence factors studied, in agreement with the observations of other authors (Archimbaud *et al.*, 2002).

In conclusion, our results confirm the presence of various virulence genes in clinical *E. faecalis* isolates and the absence of the same genes in *E. faecium* isolates, with the exception of *esp*. We observed that the *E. faecium* isolates analysed are enriched in *esp*, a phenomenon that has been described previously for *E. faecalis* (Shankar *et al.*, 1999) and only recently for *E. faecium* of nosocomial origin (Eaton & Gasson, 2001; Willems *et al.*, 2001; Woodford *et al.*, 2001), but we were not able to correlate the presence of *esp* with the capacity to form biofilms or to adhere to epithelial cells. Further studies are necessary in order to analyse the association and role of virulence genes in the pathogenicity of *E. faecium*.

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