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Development of a Multiplex PCR for the Detection of *asa1*, *gelE*, *cylA*, *esp*, and *hyl* Genes in Enterococci and Survey for Virulence Determinants among European Hospital Isolates of *Enterococcus faecium*

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A multiplex PCR for the simultaneous detection of five virulence genes (*asa1*, *gelE*, *cylA*, *esp*, and *hyl*) in enterococci was developed. The presence of these genes was investigated in 153 clinical and 118 fecal *Enterococcus faecium* isolates from inpatients at an increased risk of developing infections (such as patients in intensive care units and hematology wards) from 13 hospitals in eight European countries. Of the 271 *E. faecium* isolates, 135 were vancomycin resistant *E. faecium* (VREF) isolates and 136 were vancomycin susceptible *E. faecium* (VSEF) isolates. Susceptibilities to ampicillin, gentamicin, streptomycin, vancomycin, teicoplanin, ramoplanin, quinupristin-dalfopristin, and linezolid were tested by the microdilution method. Overall, the prevalence of *esp* was significantly higher ($P = 0.03$) in clinical VREF isolates (92%) than in fecal VREF isolates (73%). In Italy, the prevalence of *esp* was significantly higher ($P = 0.02$) in VREF isolates (91%) than in VSEF isolates (68%), whereas in the United Kingdom, *hyl* was significantly more prevalent ($P = 0.01$) in VREF isolates (71%) than in VSEF isolates (29%). No significant differences were found for the other countries. Pulsed-field gel electrophoresis was used to check the clonality among the strains tested and showed the spread of two center-specific (*esp*-positive) VREF clones in Italy and one center-specific (*hyl*-positive) clone in the United Kingdom. These clones were resistant to ampicillin, gentamicin, and streptomycin. The multiplex PCR reported in this study is a convenient and rapid method for the simultaneous detection of the virulence genes *asa1*, *gelE*, *cylA*, *esp*, and *hyl* in enterococci. Molecular analysis showed the intrahospital spread of *esp*-positive VREF clones (in Italy) and *hyl*-positive VREF clones (in the United Kingdom); the role of *hyl* remains to be elucidated.

Enterococci form part of the normal flora of both the human and the animal gastrointestinal tract. However, vancomycin-resistant enterococci have emerged as a major cause of nosocomial infections (28). Several virulence factors have been described in enterococci, for instance, aggregation substance (14), gelatinase (39), cytolysin (4), enterococcal surface protein (38), and, very recently, hyaluronidase (35). The first four virulence factors are found in *Enterococcus faecalis*, while the fourth and fifth virulence factors are specific for *Enterococcus faecium*.

Aggregation substance, encoded by *asa1*, which is carried on a plasmid, is a pheromone-inducible protein that enables the conjugative transfer of sex pheromone gene-containing plasmids through the clumping of one *Enterococcus* to another (14). As a virulence factor, aggregation substance increases bacterial adherence to renal tubular cells (26) and heart endocardial cells (18), augments internalization by intestinal epithelial cells (30), and has been shown to increase the valvular vegetation mass in an animal model of endocarditis (3).

Gelatinase, encoded by the chromosomal *gelE*, is an extracellular zinc endopeptidase that hydrolyzes collagen, gelatin, and small peptides (39) and that has been shown to exacerbate endocarditis in an animal model (17).

The production of cytolysin has also been shown to significantly worsen the severity of endocarditis (3) and endophthalmitis (24) in animal models as well as to contribute to the severity of enterococcal disease in humans (22). Cytolysin genes are carried on a plasmid or are integrated into the bacterial chromosome (23). Cytolysin consists of two components, lysin (L) and activator (A). The cytolysin operon consists of five genes, of which *cylL1*, *cylL2*, *cylM*, and *cylB* are relevant to the expression of component L, whereas *cylA* is necessary for the expression of component A (15, 21).

The enterococcal surface protein, encoded by the chromosomal *esp*, 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 (38). Enterococcal surface protein is associated with increased virulence (38), colonization and persistence in the urinary tract (37), and biofilm formation (41). A variant *esp* gene has recently been identified as a marker of highly prevalent vancomycin-resistant *E. faecium* (VREF) clones among hospitalized patients (44). However, the *esp* gene has also been

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TABLE 1. PCR primers and products for the detection of virulence genes

Gene	Virulence factor	Primer name	Oligonucleotide sequence (5′ to 3′)	Product size (bp)
asa1	Aggregation substance	ASA 11 ASA 12	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	375
gelE	Gelatinase	GEL 11 GEL 12	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	213
cylA	Cytolysin	CYT I CYT IIb	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688
esp	Enterococcal surface protein	ESP 14F ESP 12R	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	510
hyl	Hyaluronidase	HYL n1 HYL n2	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	276

detected in vancomycin-susceptible *E. faecium* (VSEF) isolates (45).

Recently, another virulence factor, hyaluronidase, was described in *E. faecium* (35). The *E. faecium* hyaluronidase, encoded by the chromosomal *hyl*, shows homology to the hyaluronidases previously described in *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (20), which are believed to contribute to invasion of the nasopharynx and pneumococcal pneumonia (2, 33).

Multiplex PCR is a rapid and convenient assay that allows simultaneous amplification of more than one locus in the same reaction and is used in both clinical and research laboratories (19). The purpose of this study was to develop a multiplex PCR for the detection of five potential virulence factors in enterococci and to investigate their presence in *E. faecium* isolates from eight European countries. Any possible correlation between these potential virulence factors, antibiotic resistance, and clonality was also explored.

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MATERIALS AND METHODS

Bacterial isolates. A total of 271 *E. faecium* isolates (153 clinical isolates and 118 fecal isolates) collected in 2001 from inpatients at increased risk of the development of infections (such as patients in intensive care units [ICUs] and hematology wards) from 13 hospitals in eight European countries (12 centers: Austria, $n = 1$; Belgium, $n = 1$; France, $n = 2$; Germany, $n = 1$; Italy, $n = 3$; The Netherlands, $n = 1$; Spain, $n = 2$; United Kingdom, $n = 1$) were included in this study. In total, 135 *E. faecium* isolates were VREF, whereas 136 were VSEF. Of the 271 strains, 115 (96 VREF isolates and 19 VSEF isolates) were isolated in Italy; 42 (28 VREF isolates and 14 VSEF isolates) were isolated in the United Kingdom; and 114 (11 VREF isolates and 103 VSEF isolates) were isolated in Austria, Belgium, France, Greece, Spain, and The Netherlands. The isolates have been identified in a previous study on ramoplanin susceptibility (16).

The reference strains used for the multiplex PCR were *E. faecalis* MMH594 (a generous gift from N. Shankar, Department of Medicinal Chemistry and Pharmaceutics, University of Oklahoma Health Sciences Center, Oklahoma City) (38) was used as a positive control strain for the detection of *asa1*, *gelE*, *cylA*, and *esp*; *E. faecium* C68 and C38 (both strains were generous gifts from L. Rice, Research and Medical Services, Louis Stokes Cleveland Veterans Affairs Medical Center, and Department of Medicine, Case Western Reserve University, Cleveland, Ohio) (35) were used as positive and negative control strains, respectively, for the detection of *hyl* (both controls were also positive for *esp*); and *E. faecalis* 217, an endocarditis isolate from The Netherlands, was used as a negative control for the detection of the virulence genes tested.

Furthermore, the multiplex PCR protocol was validated with 50 *E. faecalis* strains (35 clinical and 15 fecal strains), *E. faecalis* JH2-7 and *E. faecalis* OG1X (both strains were a generous gift from B. Murray, Center for Infectious Diseases, Department of Internal Medicine and Department of Microbiology and Molecular Genetics, University of Texas, Houston) (4), *E. faecalis* FA2-2 (a generous gift from N. Shankar) (38), *E. faecium* E-470 (a generous gift from R. Willems, University Medical Center Utrecht, Utrecht, The Netherlands) (44), and five *E. faecium* strains for which the virulence genes present were unknown to us (the five strains were sent by G. Werner from the Robert Koch Institute, Wernigerode, Germany, for use as quality control strains).

Oligonucleotide primers. The five oligonucleotide primer pairs (Eurogentec, Seraing, Belgium) used to amplify the genes *asa1*, *gelE*, *cylA*, *esp*, and *hyl* and the expected amplicon sizes are listed in Table 1. Primers were based on published primer pairs for *cylA* (4) and *esp* (44), while primers for the detection of *asa1* (GenBank accession number X17214) (14), *gelE* (GenBank accession number M37185) (39), and *hyl* (GenBank accession number AF544400) (35) were developed by using Primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Primers were designed so that the PCR products were sufficiently different in size to be distinguishable by agarose gel electrophoresis. Primer specificity was checked by a search with the BLAST program, available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Multiplex PCR. All cultures were grown on Columbia agar (Becton Dickinson, Sparks, Md.) supplemented with 5% defibrinated horse blood and were incubated at 37°C. The template DNA was prepared by suspending one loopful of bacterial cells from an overnight culture in 1 ml of Milli Q water. The bacterial suspensions were heated for 5 min at 95°C and centrifuged to remove the debris. PCR was performed in a GeneAmp PCR System 9600 (Perkin-Elmer, Wellesley, Mass.). Each 50-μl PCR mixture consisted of 5 μl of bacterial suspension; a 0.1 μM concentration (each) of primers specific for *asa1*, *gelE*, and *hyl*; a 0.2 μM concentration (each) of primers specific for *cylA* and *esp*; 25 μl of HotStarTaq master mixture (Qiagen, Hilden, Germany), which consisted of 2.5 U of HotStarTaq DNA polymerase, 1.5 mM $MgCl_2$, and 200 μM deoxynucleoside triphosphates; and an additional 1.0 mM $MgCl_2$. An initial activation step at 95°C for 15 min, during which the HotStarTaq DNA polymerase is activated, was followed by 30 cycles of denaturation (94°C for 1 min), annealing (56°C for 1 min), and extension (72°C for 1 min), followed by one cycle consisting of 10 min at 72°C. After amplification, 25 μl of the amplicon was mixed with 5 μl of gel loading buffer (50% glycerol, 0.8 mg of bromophenol blue per ml) and electrophoresed in a 1.5% pronarose D1 gel (SphaeroQ, Burgos, Spain) for 1 h at 150 V in 0.5× TBE (Tris-borate-EDTA) containing 0.05 mg of ethidium bromide per liter (positive and negative controls were included in each set of amplifications). A 100-bp DNA ladder (Invitrogen, Merelbeke, Belgium) was used as a molecular size marker.

PFGE. The clonal distribution among VREF isolates was studied as described previously (16) by pulsed-field gel electrophoresis (PFGE) by the method of Descheemaeker et al. (7). Briefly, bacterial cells from an overnight culture were imbedded in low-melting-point preparative agarose (Bio-Rad Laboratories, Naz-

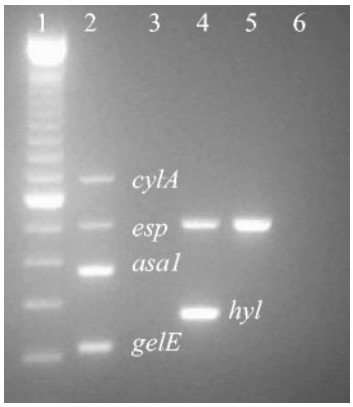

FIG. 1. Multiplex PCR of control strains. Lane 1, molecular marker (100 bp); lane 2, *E. faecalis* MMH594 (positive control for *asa1*, *gelE*, *cylA*, and *esp*); lane 3, *E. faecalis* 217 (negative control for *asa1*, *gelE*, *cylA*, and *esp*); lane 4, *E. faecium* C68 (positive control for *hyl*; also positive for *esp*); lane 5, *E. faecium* C38 (negative control for *hyl*; positive for *esp*); lane 6, negative control (no DNA added).

areth, Belgium). After cell wall and protein digestion, the plugs were digested overnight with 30 U of SmaI (MBI Fermentas, St. Leon-Rot, Germany) at 25°C. PFGE was performed with a 1% agarose gel by using a CHEF Mapper apparatus (Bio-Rad Laboratories) in 0.5× TBE buffer at 14°C and 6 V/cm. For separation, a linearly ramped switching time from 5 to 35 s was applied for 24 h. The gels were stained with ethidium bromide to detect the DNA band profiles, and the image was digitized with a Gel Doc 1000 system (Bio-Rad Laboratories). Conversion, normalization, and further analysis of the DNA band patterns were performed with GelCompar software (version 4.0b; Applied Maths, Kortrijk, Belgium), as described previously (34). The similarity between PFGE patterns was evaluated by use of the Dice coefficient and was observed visually by the detection of a maximum of three clearly visible bands.

Antimicrobial susceptibility testing. The antimicrobial susceptibilities of all strains were tested as described previously (16). They were tested for their susceptibilities to ampicillin (Sigma Chemical Co., St. Louis, Mo.), gentamicin (Sigma), streptomycin (Sigma), vancomycin (Sigma), teicoplanin (Gruppo Lepetit, Milan, Italy), ramoplanin (Vicuron Pharmaceuticals, Gerenzano, Italy), quinupristin-dalfopristin (Synercid; commercial preparation of Aventis, Milan, Italy), and linezolid (Zyvox; commercial preparation of Pharmacia, Milan, Italy) by the microdilution method, according to the guidelines of NCCLS (29). The genes responsible for resistance to vancomycin (*vanA*, *vanB*, *vanC1*, and *vanC2*) were detected by PCR, as described previously (9).

Statistical analysis. Chi-square analysis of contingency tables and Fisher's exact test were used for statistical analysis. A P value <0.05 was considered statistically significant.

RESULTS

Development of multiplex PCR. Template DNA was prepared by using cell suspensions of only a few colonies since thicker cell suspensions, as described by other investigators (25, 31), increased the amplicon intensity, which has also been noticed by others (13, 32). Although at first the primer concentrations used for the detection of each gene were the same, increasing the primer concentrations for *cylA* and *esp* helped visualize the expected PCR products much more consistently. Moreover, a higher concentration of Mg^{2+} was needed to optimize the intensities of the band patterns generated.

As shown in Fig. 1, the PCR product of the expected size for each of the four control strains was observed. The *E. faecalis* positive control strain, strain MMH594 (Fig. 1, lane 2), yielded four gene-specific products (*asa1*, *gelE*, *cylA*, and *esp*), while no products were generated from the *E. faecalis* negative control strain, strain 217 (Fig. 1, lane 3). The *E. faecium* positive control strain for *hyl*, strain C68 (Fig. 1, lane 4), yielded two gene-specific products (*esp* and *hyl*), while only *esp* was generated from the *E. faecium* negative control strain for *hyl*, strain C38 (Fig. 1, lane 5). Each multiplex PCR assay was performed with a negative control containing all the reagents but no DNA template (Fig. 1, lane 6).

The 53 *E. faecalis* strains contained one, two, three, all, or none of the virulence genes tested for in this study, which was identical to the results generated by uniplex PCRs for each of the virulence genes tested for. Upon comparison of the two primer sets specific for *esp* reported so far (38, 44), only those described by Willems et al. (44) could consistently detect *esp* in two of six *E. faecium* isolates.

Multiplex PCR survey for virulence factors among *E. faecium* isolates. We tested 271 *E. faecium* strains for the presence of five virulence factors. The genes *asa1*, *gelE*, and *cylA* were not detected. Of all 271 (VREF and VSEF) strains, 176 (65%) were positive for *esp* and 45 (17%) were positive for *hyl*. The prevalence of *esp* was significantly higher ($P = 0.04$) among clinical VREF isolates (24 of 26 [92%]) than among fecal VREF isolates (80 of 109 [73%]). The *esp* gene was significantly more prevalent ($P < 0.0001$) among the VREF isolates than among the VSEF isolates: 104 of 135 (77%) VREF isolates versus 72 of 136 (53%) VSEF isolates. A significant difference in the prevalence of *esp* ($P = 0.02$) was found among the 115 Italian isolates (96 VREF isolates and 19 VSEF isolates): 87 of 96 (91%) VREF isolates versus 13 of 19 (68%) VSEF isolates possessed this gene. No significant difference in the prevalence of *esp* was observed between United Kingdom VSEF isolates and VREF isolates. The *esp* gene was significantly more common ($P < 0.0001$) among ampicillin-resistant VREF isolates (93 of 109 [85%]) than among ampicillin-susceptible VREF isolates (11 of 26 [42%]). Among the *esp*-positive VREF isolates, ampicillin resistance was seen in 93 of 104 (89%), of which 82 of 87 (94%) were Italian *esp*-positive VREF isolates and 9 of 15 (60%) were *esp*-positive United Kingdom VREF isolates.

The *hyl* gene was found in 7 of 26 (27%) of the clinical VREF isolates, whereas it was found in 15 of 109 (14%) of the fecal VREF isolates ($P = 0.1$). No significant difference in the prevalence of *hyl* was seen between the Italian VSEF and VREF isolates. A significant difference ($P = 0.01$) in the prevalence of *hyl* was observed among the 42 United Kingdom isolates (28 VREF isolates and 14 VSEF isolates): 20 of 28 (71%) VREF isolates versus 4 of 14 (29%) VSEF isolates were found to be positive for this gene; however, no significant difference in the prevalence of *hyl* was observed between the Italian VREF and VSEF isolates. The *hyl* gene was significantly more common ($P < 0.0001$) among ampicillin-resistant VREF isolates (16 of 109 [15%]) than among ampicillin-susceptible VREF isolates (6 of 26 [2%]). Ampicillin resistance was seen in 16 of 22 (73%) of the *hyl*-positive VREF isolates, of which the only Italian *hyl*-positive VREF isolates and 14 of 20 (70%) United Kingdom *hyl*-positive VREF isolates were resistant.

Of the 114 isolates (11 VREF isolates and 103 VSEF isolates) collected from Austria, Belgium, France, Greece, Spain, and The Netherlands, *esp* was detected in 51 (45%) isolates (2 of 11 [18%] VREF isolates and 49 of 103 [48%] VSEF isolates)

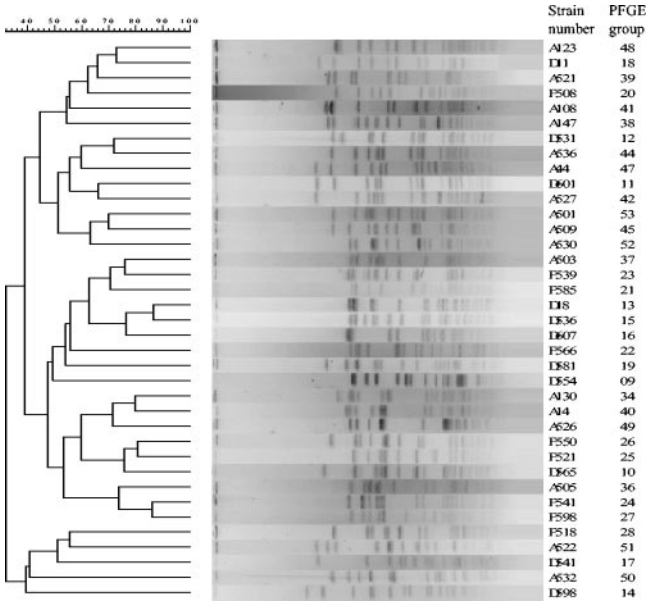

FIG. 2. Dendrogram of PFGE patterns.

and *hyl* was present in 19 (17%) isolates (1 of 11 [9%] VREF isolates and 18 of 103 [18%] VSEF isolates).

PFGE typing, susceptibility testing, and the presence of virulence determinants. PFGE groups were defined as described previously (40). A dendrogram is shown in Fig. 2. PFGE revealed the spread of two center-specific *esp*-positive VREF clones in Italy (referred to as centers D and F below) and one center-specific *hyl*-positive VREF clone in the United Kingdom (referred to as center A below); these clones were resistant to ampicillin, gentamicin, and streptomycin. In center D in Italy, among the 58 VREF isolates collected, PFGE revealed 11 groups, all of which were of the VanA phenotype (Table 2). Except for the isolates belonging to PFGE groups 9 and 17, all isolates were positive for *esp*, of which group 11 represented the largest number of isolates. In center F in Italy, among the 36 VREF isolates collected, PFGE revealed 9 groups, all of which were of the VanA phenotype (Table 2). The isolates of PFGE group 20 were positive for *esp* and *hyl*, while the isolates of PFGE groups 25, 26, 27, and 28, of which group 26 contained the largest number of isolates, were positive only for *esp*. The other isolates (PFGE groups 21, 22, 23, and 24) did not show the presence of any virulence factors. All of the Italian isolates were highly resistant to ampicillin, gentamicin, and streptomycin. In center A in the United Kingdom, among the 28 VREF isolates collected, 24 of the VanA phenotype and 4 of the VanB phenotype, PFGE revealed 17 groups, of which group 49 contained the largest number of isolates (Table 2). The isolates of PFGE groups 34, 39, 40, 41, 47, 48, 49, 50, 51, and 52 were positive for *esp* and *hyl*; the isolates of PFGE group 37 were positive only for *hyl*; and the isolates of PFGE groups 38 and 53 were positive only for *esp*. No virulence factors were detected in the other groups (groups 36, 42, 44, and 45). All the United Kingdom isolates were resistant to ampicillin, and of these, 19 and 21 isolates also showed resistance to gentamicin and streptomycin, respectively. One Italian strain of PFGE group 16 and one United Kingdom strain of PFGE group 49 showed *esp* positivity, while the other isolates of the same group did not (Table 2). Finally, one strain was resistant to linezolid (8 mg/liter); this strain was isolated in the United Kingdom, belonged to PFGE group 49, and was positive for *hyl*. Ramoplanin was active against all strains of VREF, with an MIC at which 90% of isolates are inhibited of 0.5 mg/liter for the clinical isolates.

DISCUSSION

A multiplex PCR developed for the simultaneous detection of enterococcal genes that encode for aggregation substance (*asa1*), gelatinase (*gelE*), cytolysin (*cylA*), enterococcal surface protein (*esp*), and hyaluronidase (*hyl*) has not been described before. The multiplex protocol for these five genes provides a reliable and rapid alternative to phenotypic testing and uniplex PCRs. Moreover, the use of 5 μl of a heat-treated bacterial suspension as the DNA template is a time-saving step compared to the amount of time required for DNA preparation, increasing the feasibility of the technique.

We surveyed European *E. faecium* isolates for the presence of these genes. The *asa1*, *gelE*, and *cylA* genes were not detected in any of the 271 *E. faecium* isolates, which is in agreement with the results reported by other investigators who also tested *E. faecium* strains for the presence of one or more of these genes (4, 8, 10, 12, 36). However, Eaton and Gasson (10) found one *gelE*-positive *E. faecium* isolate without phenotypic gelatinase activity, and Elsner et al. (12) found *asa1* among 13% of clinical *E. faecium* isolates, but they used hybridization.

The *esp* gene was detected in 65% of *E. faecium* isolates, in accordance with the findings of other studies (10, 11), which identified the *esp* gene in about 80% of *E. faecium* strains. However, this is in contrast to the findings of Shankar et al. (38), who reported the absence of *esp* in *E. faecium*. We detected the *esp* gene in a significantly higher number ($P < 0.0001$) of VREF strains (77%) than VSEF strains (53%). Previous studies on the incidence of *esp* in VREF and VSEF are contradictory: some studies (44) indicated a higher prevalence of *esp* among VREF strains than among VSEF strains, other studies showed the opposite (5, 8, 27), whereas again in others an equal distribution of the *esp* gene was found among VREF and VSEF strains (35, 45). Interestingly, we found that 91% of the Italian VREF strains harbored the *esp* gene. This is in contrast to the results of Baldassarri et al. (1), who found this gene in 33% of clinical VSEF isolates. The prevalence of *esp* in the present study was significantly higher ($P = 0.04$) in the clinical VREF isolates (92%) than in the fecal VREF isolates (73%). This is in agreement with the results of Rice et al. (35), who also reported a higher prevalence of *esp* among clinical isolates compared with that among fecal isolates. These results suggest a possible role of *esp* in the pathogenicity of enterococci.

Analysis of the clonality *E. faecium* strains harboring the *esp* gene showed that 5 of 9 clonal types (56%) from center D in Italy, 8 of 11 clonal types (73%) from center F in Italy, and 11 of 19 clonal types (58%) from center A in the United Kingdom were *esp* positive. The clonal distribution of *E. faecium* strains harboring *esp* has also been reported by Coque et al. (5, 6), while Willems et al. (44) described a larger number of *esp*-

TABLE 2. Vancomycin-resistant *E. faecium* strains in centers D and F in Italy and center A in the United Kingdom

Center/country	PFGE group	No. of isolates	Ward of isolation	No. of specimens of the following type[a]:		VanA or VanB phenotype	Detection of:	
				Clinical	Fecal		*esp*	*hyl*
D/Italy	9	1	ICU		1	VanA	−	−
D/Italy	10	1	ICU		1	VanA	+	−
D/Italy	11	30	ICU	7 (3)	23	VanA	+	−
D/Italy	12	7	ICU	1 (1)	6	VanA	+	−
D/Italy	13	7	ICU	2 (2)	5	VanA	+	−
D/Italy	14	1	ICU		1	VanA	+	−
D/Italy	15	1	ICU		1	VanA	+	−
D/Italy	16	4	ICU	1	3	VanA	+[b]	−
D/Italy	17	1	ICU		1	VanA	−	−
D/Italy	18	1	Surgery	1		VanA	+	−
D/Italy	19	4	ICU	1	3	VanA	+	−
F/Italy	20	1	Medicine		1	VanA	+	+
F/Italy	21	1	Medicine		1	VanA	−	−
F/Italy	22	1	Outpatient		1	VanA	−	−
F/Italy	23	1	Hematology		1	VanA	−	−
F/Italy	24	1	Hematology		1	VanA	−	−
F/Italy	25	9	Medicine, $n = 6$; urology, $n = 1$; hematology, $n = 1$; surgery, $n = 1$	1	8	VanA	+	−
F/Italy	26	18	Medicine, $n = 9$; hematology, $n = 8$; surgery, $n = 1$	2 (1)	16	VanA	+	−
F/Italy	27	1	Surgery		1	VanA	+	−
F/Italy	28	3	Hematology	1 (1)	2	VanA	+	−
A/United Kingdom	34	1	ICU	1		VanA	+	+
A/United Kingdom	36	1	Pediatrics		1	VanA	−	−
A/United Kingdom	37	1	Pediatrics		1	VanA	−	+
A/United Kingdom	38	1	Pediatrics	1 (1)		VanA	+	−
A/United Kingdom	39	1	Hematology		1	VanB	+	+
A/United Kingdom	40	1	ICU	1		VanA	+	+
A/United Kingdom	41	1	Medicine	1		VanB	+	+
A/United Kingdom	42	1	Pediatrics		1	VanA	−	−
A/United Kingdom	44	1	Hematology		1	VanA	−	−
A/United Kingdom	45	1	Hematology		1	VanA	−	−
A/United Kingdom	47	2	Urology, $n = 1$; solid organ transplant, $n = 1$	2		VanA	+	+
A/United Kingdom	48	1	Medicine	1 (1)		VanA	+	+
A/United Kingdom	49	9	Hematology		9	VanA	+[c]	+
A/United Kingdom	50	1	Hematology		1	VanB	+	+
A/United Kingdom	51	1	Hematology		1	VanB	+	+
A/United Kingdom	52	1	Hematology		1	VanA	+	+
A/United Kingdom	53	3	Hematology		3	VanA	+	−

[a] Values in parentheses are the number of isolates from blood.
[b] One of four isolates.
[c] One of nine isolates.

positive clones and, thus, reported a distribution of *esp* more heterogeneous than that found in our study.

Deviating results were found for one Italian strain of PFGE group 16 and one United Kingdom strain of group 49. Both strains were found to be *esp* positive, while other strains belonging to the same group were *esp* negative. This difference might be explained by the fact that the restriction enzyme used for PFGE, SmaI, does not recognize restriction sites in the *esp* gene. Moreover, SmaI generates segments of kilobase pairs, while the multiplex PCR generates segments of base pairs, and because of the difference in the lengths of the fragments generated by PFGE and PCR, it is unlikely that the virulence gene will be observed by PFGE. Our findings could be further explored by using a second restriction enzyme, which might be able to detect the differences. According to Waar et al. (43), *E. faecalis* isolates are clonal if they reveal a similarity of ≥90% by amplified fragment length polymorphism analysis and an identical pattern of virulence factors. On the basis of these conclusions, the clones reported in our study may have to be reclassified in a different PFGE cluster.

The *hyl* gene was detected in 17% of the 271 *E. faecium* isolates collected in eight European countries, which is in contrast to the findings of Rice et al. (35), who detected *hyl* in only 3% of the European clinical isolates. We found the *hyl* gene among 16% of the 135 VREF isolates and 17% of the 136 VSEF isolates. Rice et al. (35) found the *hyl* gene only in European VREF isolates and in none of the European VSEF isolates included in their study. Moreover, our study showed that the *hyl* gene is even more prevalent among the United Kingdom VREF isolates (71%) than among the U.S. VREF isolates (39%) described by Rice et al. (35).

The *esp* and *hyl* genes were significantly more common

among ampicillin-resistant VREF isolates than among ampicillin-susceptible VREF isolates, which is in accordance with the findings of other studies (5, 6, 27, 42).

Finally, we found that 34 of 45 (76%) *hyl*-positive strains were also *esp* positive, which is in accordance with the findings of Rice et al. (35), who also described the combined presence of *hyl* and *esp* in >90% of the strains that they tested. On the contrary, Coque et al. (6) found only 4% of their isolates to be positive for *esp* and *hyl*.

In conclusion, the multiplex PCR developed and described herein is a convenient and rapid method for the simultaneous detection of five potential virulence genes, *asa1*, *gelE*, *cylA*, *esp*, and *hyl*, in enterococci. Molecular analysis showed the intrahospital spread of *esp*-positive VREF clones (in Italy) and of *hyl*-positive VREF clones (in the United Kingdom); the role of *hyl* remains to be elucidated.

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