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Survey of Enterococcal Susceptibility Patterns in Belgium

P. VANDAMME,[1,2]* E. VERCAUTEREN,[1] C. LAMMENS,[1] N. PENSART,[3] M. IEVEN,[1] B. POT,[2] R. LECLERCQ,[3] AND H. GOOSSENS[1]

Department of Microbiology, University Hospital UIA, Antwerp,[1] and University of Ghent, Ghent,[2] Belgium, and Department of Microbiology, Henri Mondor Hospital Créteil, France[3]

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A national surveillance study was performed in order to identify the enterococci causing important infections, to determine their susceptibilities to first-choice agents for treatment, and to characterize the phenotypes and genotypes of the glycopeptide-resistant strains. A total of 472 isolates were collected between 15 January and 15 April 1993. The ability of the API rapid ID 32 STREP gallery to identify enterococci was evaluated. The majority of the Belgian enterococci were identified as *E. faecalis* (89.4%). *E. faecium* and other enterococci were present in small percentages only (9.1 and 1.5%, respectively). The API rapid ID 32 STREP system identified 88.6% of the strains with an excellent or very good identification score. For the majority of the strains with uncertain identification scores, the results of a single test only were aberrant. Only 2.3% of the strains remained unidentified. High-level aminoglycoside resistance was widespread in *E. faecalis* (streptomycin, 50.8%; gentamicin, 8.7%), and the emergence of ciprofloxacin resistance was found to be associated with aminoglycoside resistance. *E. faecium* is generally more resistant to a wide range of antibiotics, but glycopeptide-resistant strains (1.5%) have not yet become widespread.

Enterococci have become notorious nosocomial pathogens, in spite of their low level of virulence (12, 22, 39). The increasing importance of these bacteria is largely due to their resistance to many antimicrobial agents, including β-lactam antibiotics, glycopeptides, and aminoglycosides (12, 15, 21). In particular, multiply resistant *Enterococcus faecium* strains carrying intrinsic and acquired resistance determinants pose life-threatening problems (12, 31, 39). The endogenous flora has long been considered the source of these enterococcal infections, with person-to-person spread being the mode of transmission within hospitals (15, 23). Moreover, glycopeptide-resistant *E. faecium* strains have recently been isolated from farm animals (3, 17) and environmental sources (17, 18), which enhances significantly the number of potential reservoirs for infection.

A national surveillance study was performed in order to identify the enterococcal species causing important infections, to determine their susceptibilities to first-choice agents for treatment, and to characterize the phenotypes and genotypes of the glycopeptide-resistant strains.

MATERIALS AND METHODS

Collection of strains in the reference center. Sixteen participating Belgian laboratories were asked to collect 30 consecutive enterococcal strains prospectively between 15 January and 15 April 1993. A maximum of one strain per patient and 10 urogenital isolates per center were allowed.

The following hospitals were included in the study: all Belgian university hospitals (seven hospitals) as well as nine other Belgian hospitals. The other nine hospitals were selected because they are all very large institutions (on the basis of the number of beds) and because a clinical pathologist with a good knowledge of microbiology was available in the centers. The 16 hospitals are geographically spread over Belgium (7 hospitals from the Flemish community, 3 hospitals from the French community, and 6 hospitals from Brussels).

Identification of strains. Identification of the strains to the genus level was performed by using the following characteristics: reaction on Gram staining, cellular morphology, presence of Lancefield group D antigen, growth and blackening of bile-esculin agar, growth in the presence of 6.5% NaCl and at 45°C, presence of catalase and pyrrolidonyl arylamidase (Rosco Diagnostica) activities, and acidification of glucose with the production of gas.

All enterococci were identified to the species level by using the API rapid ID 32 STREP gallery (bioMérieux) and the software supplied by the manufacturer. Three categories of identification scores were distinguished: (i) excellent or very good identification, (ii) uncertain identification, and (iii) no identification. Some strains (see below) were also identified by the conventional method of Facklam and Collins (13) and polyacrylamide gel electrophoresis (PAGE) of whole-cell proteins. For protein analysis, the strains were grown for 24 h at 37°C on brain heart infusion agar (BHI; Difco) and were incubated in a microaerobic atmosphere containing 5% O_2, 10% CO_2, and 85% N_2. PAGE of whole-cell proteins, densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis were performed as described by Pot et al. (30). Strains were identified by using a database comprising the protein patterns of more than 500 strains representing all enterococcal species.

Susceptibility testing. Susceptibility was determined by the criteria recommended by the National Committee for Clinical Laboratory Standards (24, 25). The following drugs were tested: penicillin (Continental Pharma), ampicillin (Bristol-Myers Squibb), ciprofloxacin (Bayer), vancomycin (Eli Lilly), and teicoplanin (Marion Merrell Dow); *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Enterococcus faecalis* ATCC 29212 were used for quality control. MICs were determined by the broth microdilution method; in addition, disk susceptibility tests were performed for vancomycin (30 μg; bioMérieux), teicoplanin (30 μg; bioMérieux), and piperacillin (100 μg; BBL Microbiology Systems).

High-level resistance toward gentamicin (Schering-Plough), streptomycin (Sigma), and aminoglycosides was determined on Mueller-Hinton agar (BBL Microbiology Systems) supplemented with streptomycin (2,000 μg/ml) and gentamicin (500 and 2,000 μg/ml) as described by Sahm and Torres (32).

Finally, resistance to vancomycin was also registered on BHI agar containing 6 μg of vancomycin per ml as described by Swenson et al. (35). The presence of β-lactamase was determined with cefinase disks (BBL Microbiology Systems).

Genotypic detection of resistance to glycopeptides. The determinants of resistance to glycopeptides, *vanA*, *vanB*, and *vanC1*, were detected by a PCR-based technique as described by Dutka-Malen et al. (11). In strains positive for amplification with primers specific for *vanA*, the presence of Tn*1546*-related sequences was further tested. The structure of putative Tn*1546*-like elements was analyzed by hybridization and PCR. For hybridization experiments, enterococcal DNAs were prepared as described previously (2) and were digested either with *Bgl*II or with *Hin*dIII and *Eco*RI enzymes. The digested DNAs were resolved by electrophoresis in 0.8% agarose gels and were transferred to nylon membranes (Hybond N; Amersham, Les Ulis, France). The 5-kb *Eco*RI-*Eco*RV (probe A) and 1.2-kb *Xba*I (probe C) fragments corresponding to the left and right ends of Tn*1546*, respectively, were used as probes in hybridization experiments as described previously by Arthur et al. (2). The probes were labelled by the random priming method (oligonucleotide kit; Pharmacia) with [α-^{32}P]dCTP (3,000 Ci/mmol; ICN Biomedicals, Inc.). Hybridization was performed overnight under stringent conditions (60°C in 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.25% sodium dodecyl sulfate, and 0.25% skim milk). For PCR analysis, a set of six pairs of oligonucleotides was used to amplify overlapping

* Corresponding author. Mailing address: Universitaire Instelling Antwerpen, Universiteitsplein 1, B-2610 Wilrijk, Belgium. Phone and fax: (32)3.820.2663. Electronic mail address: Peter.Vandamme@rug.ac.be.

TABLE 1. Species-level identification results for 472 enterococci

API identification score	No. of strains	Identification result by:		
		API rapid ID 32 STREP	Conventional scheme	Protein electrophoresis[a]
Excellent or very good	379	*E. faecalis*	ND[b]	ND
	31	*E. faecium*	*E. faecium*	*E. faecium*
	2	*E. gallinarum*	*E. gallinarum*	*E. gallinarum*
	1	*E. avium*	*E. avium*	*E. avium*
	1	*E. casseliflavus*	*E. casseliflavus*	*E. casseliflavus*
				E. flavescens group
	1	*E. hirae*	*E. hirae*	*E. hirae*
	2	*E. gallinarum*	*E. faecium*	*E. faecium*
	1	*E. casseliflavus*	*E. faecium*	*E. faecium*
Uncertain	37	*E. faecalis*	*E. faecalis*	*E. faecalis*[c]
	1	*E. faecium*	*E. faecium*	*E. faecium*
	1	*E. gallinarum*	*E. gallinarum*	*E. gallinarum*
	1	*E. avium*	*E. pseudoavium*	*E. avium*
	1	*E. faecium*	*E. faecalis*	*E. faecalis*[d]
	1	*E. casseliflavus*	*E. faecalis*	*E. faecalis*
	1	*E. durans*	*E. durans*	*E. faecium*
Unidentified	4		*E. faecalis*	*E. faecalis*
	6		*E. faecium*	*E. faecium*
	1		*E. hirae*	*E. faecium*

[a] Considered the identification standard when it was performed.
[b] ND, not determined.
[c] Only the seven strains which had aberrant results by more than one test in the API gallery were examined.
[d] This strain had an aberrant protein pattern.

fragments internal to most of Tn*1546*, as described by Arthur et al. (2). *E. faecium* BM4147 harboring Tn*1456* carried by plasmid pIP816 and *E. faecium* BM4147-1 (a derivative of BM4147 susceptible to glycopeptides) were used as positive and negative controls, respectively, in the PCR and hybridization experiments.

Genotyping. Clonal distribution among the vancomycin-resistant strains was studied by pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD) typing.

For PFGE, genomic DNA was prepared in agarose plugs as described previously by Murray et al. (23). The plugs were incubated in 3 ml of lysis solution (6 mM Tris HCl [pH 7.6], 1 M NaCl, 100 mM EDTA [pH 7.5], 0.5% Brij 58, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, 20 μg of RNase per ml, 1 mg of lysozyme per ml, 50 μg of lysostaphin per ml) for 24 h at 37°C. The solution was replaced by 3 ml of EPS solution (0.5 M EDTA [pH 9], 50 μg of proteinase K per ml, 1% sodium lauroyl sarcosine), and the mixture was incubated for 24 h at 50°C and then washed three times with 5 ml of TE buffer (10 mM Tris HCl [pH 7.6], 1 mM EDTA [pH 7.6]). A part of the plug was digested with 25 IU of *Sma*I restriction enzyme (Pharmacia, Saint Quentin en Yvelines, France) per ml and was incubated for at least 6 h at 25°C. The DNA fragments were separated in a 1.2% agarose gel that was prepared and run in 0.5× Tris-borate-EDTA buffer in a contour-clamped homogeneous field apparatus (CHEF-DR2; Bio-Rad, Ivry sur Seine, France). The parameters for electrophoresis were 200 V for 20 h, with pulse times ramped from 5 to 35 s. Profiles which differed only by three or fewer restriction fragments were classified as the same type (14).

For RAPD analyses, DNA was prepared by the rapid procedure described by Pitcher et al. (27). DNA amplification with the random primers D14307, D11344, and D8635 (1) was performed as described previously (37), except that the reaction buffer consisted of 50 mM KCl, 10 mM Tris HCl (pH 9.0), 2.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.01% gelatin. Strains were considered to belong to different types as soon as a single DNA fragment was different.

RESULTS

Collection of strains. A total of 472 strains were collected: 179 (37.9%) strains were isolated from wounds, 79 (16.7%) were from blood, 41 (8.7%) were from urine, 28 (5.9%) were from pus, 20 (4.2%) were from bile, 18 each (3.8%) were from skin and stool specimens, 15 (3.2%) were from ear specimens, 12 (2.5%) were from abdominal or peritoneal fluids, 10 (2.1%) were from bronchial or tracheal fluids, and 7 each (1.5%) were from throat, catheter, and drainage specimens. Finally, 31 strains were isolated from various additional sources, with each strain accounting for less than 1% of the strains tested.

Identification of strains. All isolates were gram-positive coccobacilli forming short chains. All strains grew on and blackened bile-esculin agar and grew in the presence of 6.5% NaCl (except for one strain) and at 45°C (except for four strains). All were catalase negative, and none produced gas from glucose. All except three strains possessed the Lancefield group D antigen and produced pyrrolidonyl arylamidase activity.

The API identification score was excellent or very good for 418 strains (88.6%) (Table 1); species identity was uncertain for 43 strains (9.1%), and 11 strains (2.3%) remained unidentified. Strains other than *E. faecalis* with excellent or very good identification scores, strains with uncertain species identity (see below), and unidentified strains were reexamined by using the conventional phenotypic identification scheme and whole-cell protein analysis.

The identification of the 31 *E. faecium* strains with an excellent identification score was confirmed by both of the other methods. Similarly, the identification of five of the eight non-*E. faecalis*, non-*E. faecium* strains with excellent identification scores was again confirmed by both of the other methods (Table 1); three strains, however (two *E. gallinarum* strains and one *E. casseliflavus* strain) were identified as *E. faecium* by both of the other techniques (Table 1).

Thirty-seven *E. faecalis* isolates had uncertain identification scores. The identification of all 37 strains was confirmed by the conventional scheme (Table 1). Of these 37 strains, 30 had only a single discordant test result in the API gallery, which was acidification of cyclodextrin (CDEX) for 13 strains and the presence of alanyl-phenylalanyl-proline arylamidase activity (APAA) for 11 strains. According to the database, these characteristics are present (CDEX) or absent (APPA) in 100% of the *E. faecalis* strains. Changing the outcomes of these tests resulted in excellent identification scores. The remaining seven

TABLE 2. Resistance patterns of the 472 enterococci tested[a]

Antibiotic	No. (%) of resistant strains		
	E. faecalis (422 strains)	*E. faecium* (43 strains)	Total (472 strains)
Penicillin	0	18 (41.9)	18 (3.8)
Ampicillin	0	10 (23.3)	10 (2.1)
Ciprofloxacin	43 (10.2)	11 (25.6)	54 (11.4)
Vancomycin	0	7 (16.3)	7 (1.5)
Teicoplanin	0	7 (16.3)	7 (1.5)
Gentamicin[b]	39 (9.2)	2 (9.2)	41 (8.7)
Streptomycin[c]	217 (51.4)	23 (53.5)	242[d] (50.8)

[a] The resistance patterns were determined by the broth microdilution method for all antibiotics except gentamicin and streptomycin, for which they were determined by agar screening.
[b] Organisms that grew on medium containing 500 μg of gentamicin per ml were considered resistant.
[c] Organisms that grew on medium containing 2,000 μg of streptomycin per ml were considered resistant.
[d] Two *E. avium* strains were streptomycin resistant.

strains which had aberrant results by more than one test were also reexamined by whole-cell protein analysis, which again confirmed that these strains were *E. faecalis.*

Finally, 6 and 11 strains also had uncertain or unidentified identification scores, respectively. For 14 of these 17 strains, both the conventional scheme and whole-cell protein analysis gave the same identification result, which was *E. faecalis* for 6 strains, *E. faecium* for 7 strains, and *E. gallinarum* for 1 strain (Table 1). For only three strains were discordant results between the outcome of the conventional scheme and the outcome of protein electrophoresis registered: one strain each of *E. durans* and *E. hirae* (identification result by the conventional scheme) was identified as *E. faecium* by protein electrophoresis and one *E. pseudoavium* strain was identified as *E. avium* (Table 1). As discussed below, we consider the outcome of protein electrophoresis to be the final standard for identification.

Susceptibility testing. The susceptibility patterns of all strains tested are listed in Table 2. Seven *E. faecium* strains were resistant to glycopeptides and several other antibiotics. Two of these strains were isolated in one of the participating centers located in Brussels, two were isolated in one center in Liège, and three were isolated in different centers in Antwerp, Liège, and Brussels. All seven strains were resistant to vancomycin (MICs, at least 32 μg/ml) and teicoplanin (MICs, at least 256 μg/ml) and grew on BHI agar containing 6 μg of vancomycin per ml. Six of these strains had high-level resistance to streptomycin. Various resistance values were recorded for the β-lactam antibiotics (data not shown).

None of the strains exhibited β-lactamase activity.

Detection of the *vanA* gene and Tn*1546*-related sequences. The *vanA* gene was detected in all seven strains resistant to both vancomycin and teicoplanin. For these strains no amplification products were obtained with primers specific for *vanB* or *vanC1* (data not shown). On the basis of the comigration of bands resolved on adjacent lanes of agarose gels and hybridization, internal *Hin*dIII, *Eco*RI, and *Bgl*II fragments of Tn*1546* were present in all strains studied. Consistently, with the six pairs of primers, PCR products that comigrated with the amplification products of *E. faecium* BM4147 were obtained.

Genotyping. Two of seven strains (strains 10/14 and 10/26) exhibited identical PFGE banding patterns after digestion with *Sma*I. Both strains were isolated in the same center. All other strains were characterized by different PFGE patterns (data not shown).

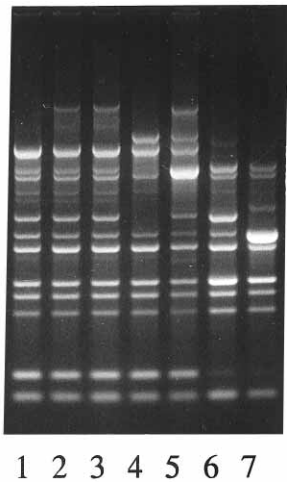

FIG. 1. DNA amplification patterns of seven vancomycin-resistant *E. faecium* strains by using oligonucleotide D14307 as the primer. Lanes: 1, strain 1/19; 2, strain 10/14; 3, strain 10/26; 4, strain 13/3; 5, strain 14/1; 6, strain 14/5; and 7, strain 15/34.

In RAPD analyses, the same two strains were identical by testing with each of the primers examined. Strain 1/19 was also identical to the former two strains by testing with primer D8635. All other strains exhibited different DNA amplification patterns with each of the primers tested. Figure 1 shows the DNA amplification patterns of the seven vancomycin-resistant strains obtained with primer D14307.

DISCUSSION

Identification of enterococci. Despite a few aberrant results, identification of enterococci to the genus level was readily achieved by using the set of physiological characters described above. The ability of the API rapid ID 32 STREP gallery and accompanying software to identify the organisms to the species level was evaluated. The majority of the strains were identified as *E. faecalis* (379 of 472 strains; 80.3%) or as *E. faecium* (31 strains; 6.6%) with an excellent or very good identification score. The identification result for these *E. faecalis* strains was not verified by other techniques, given the score and the accepted validity of the API system for the identification of this species (4). The API system identified 37 additional strains (7.8%) as *E. faecalis*, but with an uncertain identification score. Of these strains, 30 had aberrant results in a single test only. Identification of these strains by the conventional scheme and, in part, whole-cell protein analysis confirmed that they were indeed *E. faecalis*. As described above, an adaptation of the interpretation of the outcomes of these tests in the API database would probably diminish the number of uncertain identification results.

The identification of all remaining strains was reexamined by both the conventional scheme and whole-cell protein analysis. We only occasionally found discrepancies (3 of 25 strains) between the identification results obtained by the conventional scheme and those obtained by whole-cell protein analysis. It is well known that the phenotypic differentiation of *E. avium* and *E. pseudoavium* and of *E. faecium*, *E. durans*, and *E. hirae* is very difficult (9). The conventional scheme is the most widely applied method for the identification of enterococci to the species level, although several problems for species differentiation have been documented (9, 10, 26, 36, 38) and alternative identification protocols have been described, including detec-

tion of species-specific penicillin-binding proteins (40), bacteriolytic activity patterns (28), DNA probes, contour-clamped homogeneous electric field electrophoresis (10), species-specific amplification of glycopeptide resistance genes (11), and whole-cell protein electrophoresis (8, 9, 20, 36). Whole-cell protein electrophoresis has widely been documented in numerous taxonomic and identification studies to be a reference method for species delineation because a high degree of similarity in whole-cell protein content is a reflection of a high degree of DNA homology, and therefore species identity. Several reviews on the application of whole-cell protein analysis and on the correlation between protein pattern similarity and DNA homology have been published (7, 30). Therefore, we used this technique in the present study as the final standard for species identification. Interestingly, two enterococcal species, *E. casseliflavus* and *E. flavescens*, cannot be differentiated by whole-cell protein analysis (29), suggesting that they constitute a single genospecies, a finding which also emerged from other studies (9, 26). We conclude that in our collection of 472 enterococci, 422 strains belong to the species *E. faecalis*, 43 belong to *E. faecium*, 3 belong to *E. gallinarum*, 2 belong to *E. avium*, and 1 each belongs to *E. casseliflavus-E. flavescens* and *E. hirae.*

Resistance to antimicrobial agents. Important numbers of Belgian *E. faecalis* isolates acquired resistance against aminoglycosides: more than 50% of these have high-level resistance to streptomycin and about 10% have high-level resistance to gentamicin (Table 2), reflecting the distribution of aminoglycoside resistance worldwide (15). A correlation between resistance to ciprofloxacin and high-level resistance to aminoglycosides in enterococci has been reported by Schaberg et al. (33). The association between these different types of resistance determinants was confirmed in the present study, because we found significant correlations between streptomycin and ciprofloxacin resistance (chi-square = 14.0; $P = 0.0018$) and between gentamicin and ciprofloxacin resistance (chi-square = 107.2; $P < 0.0001$). In general, *E. faecalis* remains susceptible to β-lactam and glycopeptide antibiotics. Resistance due to the production of β-lactamase has been reported for *E. faecalis* and *E. faecium* strains (15), but β-lactamase production was not detected in the present collection of Belgian isolates.

The resistance patterns of *E. faecium* strains are drastically different, because high percentages of resistance to all antibiotics tested were detected. Seven of these strains acquired resistance to glycopeptides. All seven strains have the VanA phenotype and carry DNA sequences similar to that of Tn*1546* (2), indicating that Tn*1546*-related elements contributed to the dissemination of vancomycin resistance in these enterococci. Two of them were multiply resistant, yet they were susceptible to ciprofloxacin. The five remaining strains were also susceptible to ciprofloxacin. In total, about 25% of the Belgian *E. faecium* strains tested were ciprofloxacin resistant (Table 2), whereas glycopeptide-resistant *E. faecium* strains from the United States were reported to be nearly 100% (31) or 75% (16) ciprofloxacin resistant or intermediately resistant. Two glycopeptide-resistant strains isolated in the same center were clonally related, as demonstrated by PFGE, a highly sensitive technique previously used for epidemiological studies of enterococci (5, 6, 18, 31). Links between the origins of the other glycopeptide-resistant strains were not found. RAPD typing by using the random sequences D14307 (Fig. 1) and D11344 as primers substantiated the PFGE results, whereas primer D8635 was apparently less discriminatory.

Few European surveillance studies have focused on enterococci. The present study is the first such study to date in Belgium. A French multicenter study carried out in the same year (1993) found an incidence of glycopeptide-resistant enterococci of 0.76% (34). During a study in German hospitals, no glycopeptide-resistant enterococci were detected (19). A study of 2,478 strains performed by six centers in different areas of Italy between March and August 1992 showed no glycopeptide resistance among these clinical isolates. In the United Kingdom, there have been several reports of glycopeptide resistance in particular hospitals, but general data on the prevalence of these bacteria are not available. Generally, in contrast to the United States, the incidence of glycopeptide-resistant enterococci appears to be low in most European countries.

In conclusion, the majority of the enterococci examined in the present study were identified as *E. faecalis* (89.4%), outnumbering by far *E. faecium* (9.1%) and other enterococci (1.5%). These percentages are similar to the percentages reported recently by Jones et al. (16). Assuming that the fecal flora is indeed the usual source of infection (15), this could be a simple reflection of the predominance of *E. faecalis* in the endogenous flora. Most resistance phenomena, however, except for resistance to ciprofloxacin and aminoglycosides, which are somehow associated, are nearly exclusively present in *E. faecium* strains (Table 2). Streptomycin resistance in *E. faecalis* is the most relevant problem in Belgium. Glycopeptide-resistant strains have not yet become widespread. Only two of seven isolates were clonally related, and all remained susceptible to ciprofloxacin.

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