ORIGINAL ARTICLE

Clinical and molecular epidemiology of hospital *Enterococcus faecalis* isolates in eastern France

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Objective: To report on the occurrence of *Enterococcus faecalis* hospital isolates obtained during 1 year in hospitals in the Franche-Comté region of France.

Methods: Clinical isolates of *E. faecalis* of different antibiotic susceptibility phenotypes from hospitalized patients were characterized by pulsed-field gel electrophoresis. Patients with positive cultures were investigated by three case-control studies to identify risk factors for colonization/infection.

Results: The crude incidence of colonization/infection was 2.37%, and 4-day and 7-day colonization rates after admission were 10.0% and 6.36%, respectively. The rates of high-level resistance to kanamycin (HLKR) and to gentamicin (HLGR) were 47.1% and 7.1%, respectively. No isolate was resistant to glycopeptides or produced β-lactamase. The 209 hospital isolates obtained during the study yielded 98 major DNA patterns, of which two were major epidemic patterns including HLKR isolates. No single factor was significantly associated with colonization/infection by HLKR isolates. The length of hospitalization before isolation was associated with colonization by HLGR isolates.

Conclusions: The isolation frequency of *E. faecalis* strains with acquired resistance to aminoglycoside antibiotics, and the wide dissemination of resistant strains with characteristics that allow them to persist and spread, argue for further large prospective surveys of clinical isolates of *E. faecalis* in hospitals.

Key words: *Enterococcus faecalis*, high-level aminoglycoside resistance, molecular epidemiology, risk factors

INTRODUCTION

Enterococci are now firmly established as major nosocomial pathogens. The genus is the fourth most common cause of hospital-acquired infection and the third most common cause of bacteremia in the USA [1]. The treatment of choice for these infections is usually a synergistic combination of a penicillin or a glycopeptide with an aminoglycoside. The efficacy of such combinations has been compromised by the emergence of strains displaying multiple antibiotic resistance, including resistance to penicillins and glycopeptides, and high-level resistance to aminoglycosides [2]. This health threat emphasizes the importance of systematic surveillance data and strain typing to characterize the epidemiology of the evolution of resistant enterococci.

Recent clinical studies have demonstrated both intra- and inter-hospital clonal spread of vancomycin-resistant *Enterococcus faecium* (VRE), both by direct person-to-person transmission between colonized patients and medical staff, and by transmission via the environment [3–8]. Other studies report transmission of various different VRE strains [9]. Some studies have correlated the acquisition of these strains with antimicrobial pressure and particularly with the intensity

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and duration of antimicrobial therapy [5,10,11]. In contrast, epidemiologic studies have concluded that highly gentamicin-resistant *E. faecalis* isolates diverge, thereby suggesting either clonal dissemination [12,13] or the spread of related genetic determinants to clonally independent strains [14–17]. However, mutation and subsequent selection of resistant strains in genomically distinct strains can explain a part of the great genomic variability.

In June 1995, a prospective laboratory-based surveillance was initiated of resistant *E. faecalis* isolates from patients at Besançon hospital in the Franche-Comté region of France. We report the epidemiologic and microbiological characterization of the isolates obtained during the first two 1-month periods.

MATERIALS AND METHODS

Study design

Clinical cultures from all patients admitted to Besançon University Hospital were examined to identify possible cases of *E. faecalis* colonization/infection during a non-sequential study (two 1-month periods: November 1995=period A; May 1996=period B). Isolates of different antibiotic susceptibilities were characterized by pulsed-field gel electrophoresis (PFGE) and compared to isolates from other periods or origins (Table 1). Patients with positive cultures were compared by a case-control study to identify risk factors for colonization/infection with strains of different antibiotic susceptibilities.

Bacterial strains

The strains of *E. faecalis* studied were isolated from various clinical specimens. Enterococci were identified by the API 20 Strep system (BioMérieux, Lyon, France).

Table 1 Origins of isolates randomly selected for typing

Period	Phenotype categories			Origin
	Susceptible	HLKR	HLGR	
1994	0	53	14	Besançon hospital
November 1995	28	31	7	Besançon hospital
	11	10	4	Other hospitals in east France
May 1996	35	35	8	Besançon hospital
	16	20	4	Other hospitals in east France
	4	6	0	Two community laboratories in Besançon

HLKR, high-level kanamycin resistance; HLGR, high-level gentamicin resistance.

Standardized disk diffusion antimicrobial susceptibility tests were performed to determine susceptibility to erythromycin, chloramphenicol, tetracycline, amoxycillin, and piperacillin. The isolates were categorized as susceptible, intermediate or resistant according to the criteria recommended by the Comité Français de l'Antibiogramme (CFA) [18]. Isolates were tested for β-lactamase production with nitrocefin. High-level aminoglycoside resistance was determined by breakpoint screening with Mueller–Hinton agar containing kanamicin (1000 mg/L) or gentamicin (500 mg/L). High-level resistance in clinical enterococcal isolates is usually mediated by different aminoglycoside-modifying enzymes causing resistance to amikacin when kanamycin resistance is detected, and to most commercially available aminoglycosides when gentamicin resistance is detected. The MICs of vancomycin and teicoplanin were determined by the Etest method (BMD, Marne-la-Vallée, France).

DNA fingerprinting

Table 1 lists the phenotypes and origins of the isolates randomly selected for typing. PFGE of genomic DNA digested with *Sma*I was performed as described by Murray et al [19,20], using a clamped homogeneous electric-field apparatus (CHEF DRII; Bio-Rad, Hercules, CA, USA). *Staphylococcus aureus* strain NCTC 8325 DNA digested with *Sma*I was used as molecular size standard [21].

Analysis of DNA relatedness

Electrophoretic restriction patterns were analyzed by scanning photographic negatives. GelCompar software was used for cluster analysis (Applied Maths, Kortrijk, Belgium). Each strain was first compared with all other strains to calculate similarity using the Dice correlation coefficient. The strains were then grouped and the groups depicted as a dendrogram using the UPGMA clustering algorithm (unweighted pair-group method using arithmetic averages). Major restriction patterns (genotypes) were defined as patterns differing by more than three fragments with similarity coefficients of <85%, as recommended by Struelens et al [21] and Tenover et al [22]. Major genotypes were labeled with numerals and each of their variants was indicated by a suffix letter. Epidemic patterns were defined as patterns including isolates from more than three patients.

Data records

Data were collected concerning the hospital (number of beds, nature of the units, number of patients admitted) and the patients (age, sex, previous hospitalization before admission, duration of hospitalization, and antibiotic treatment within the 7 days before

isolation). Previously administered antibiotics were first analyzed as a whole group and then as separate products: prior antibiotic yes (Y)/no (N); if yes, 'potent' antibiotics for treatment of susceptible *E. faecalis* isolates Y/N, or less potent antibiotics for treatment of these isolates Y/N. Aminopenicillins, ureidopenicillins, +/− β-lactamase inhibitor, imipenem, aminoglycosides, tetracycline, erythromycin and other macrolides, and glycopeptides, were defined as 'potent' anti-*E. faecalis* antibiotics. Cephalosporins, methoxypenicillins, fluoroquinolones, metronidazole and fusidic acid were defined as less potent anti-*E. faecalis* antibiotics.

Definitions

Clinical *E. faecalis* isolates were classified as community acquired if the sample that was cultured positive was obtained within the first 24 h of admission from patients who were not hospitalized during the 48 h before isolation. Known clinical features were collected to differentiate colonization and infection. Colonization/infection was defined on the bacteriologic results of the analyses of clinical specimens.

Incidence and occurrence of enterococcal colonization or infection

The main endpoints were the incidences of *E. faecalis* colonization and infection. First, the crude incidence was estimated as the total number of cases of *E. faecalis* colonization/infection divided by the total number of exposed patients. Second, time-failure methods were used to take into account the various lengths of exposure in the hospital, and to compute the hazard function which estimates the instantaneous risk of developing colonization within fixed time intervals. The time required for colonization was calculated from the date of hospital admission within a maximum observation time in the hospital of 120 days. These estimations were based on the Kaplan–Meier method [23] and actuarial life table methods [24]. The time required for colonization was compared for strains with PFGE patterns found only in single patients ('unique' patterns) against strains with patterns found in several patients ('multiple' patterns), by the Kaplan–Meier method; prognostic values were assessed by the log-rank test at the 5% level [25].

Clinical epidemiology

To assess risk factors for colonization/infection with resistant *E. faecalis*, three case-control studies were performed: first, to identify risk factors for colonization/infection with HLGR *E. faecalis*; second, to identify risk factors for colonization/infection with HLKR *E. faecalis*; and third, to identify risk factors for colonization/infection with HLKR *E. faecalis* with a PFGE pattern characteristic of an epidemic strain. Three separate control groups were used: for the first and second studies, controls were all patients colonized or infected with susceptible *E. faecalis* isolates; for the third study, controls were patients colonized or infected with HLKR *E. faecalis* with unique patterns. Eight variables were studied as risk factors: age, sex, previous hospitalization in another unit, duration of hospitalization before colonization with *E. faecalis*, unit of hospitalization, antimicrobial therapy before colonization, and administration of potent antibiotics and less potent antibiotics before colonization. Univariate logistic regressions were performed to identify risk factors. Odds ratios were estimated by exponentiation of regression coefficients and calculation of 95% confidence intervals (CIs). The two first statistical analyses were performed using BMDP software packages. The third analysis (to identify risk factors for colonization/infection with HLKR *E. faecalis* with a unique PFGE pattern) was performed with the LogXact computer package [26] by exact logistic regression.

RESULTS

Incidence of *E. faecalis* colonization

During the study periods, 9152 patients were admitted to Besançon University Hospital for a total of 61 169 days of hospitalization. Colonization or infection with *E. faecalis* occurred in 217 of the 9152 patients, giving a crude incidence estimated at 2.37% (CI 95% = [2.06–2.68]). Eight of the 217 patients had two colonizations/infections (on two different body sites), giving a crude incidence of 3.67 colonizations/infections per 1000 days of hospitalization. There was no significant difference between the incidences of the two periods: 101 of the 4572 patients of period A (2.22%) and 116 of the 4580 patients of period B (2.53%) (RR = 0.93; CI 95% = [0.80–1.07]; $p = 0.27$). Among the 225 colonizations/infections, 54 (24%) were community-acquired. In total, 143 (63.6%) patients had isolates cultured from urinary tract specimens, 28 (12.4%) from superficial swabs, seven (3.1%) from a surgical wound, five (2.2%) from blood, and 42 (18.7%) from other specimens.

Occurrence of colonization/infection

The risk of colonization with *E. faecalis* over time was estimated to be 0.0935 within the first week, 0.0635 within the second week, and 0.0621 within the third week. The 4-day and 7-day Kaplan–Meier rates of colonization with *E. faecalis* were estimated to be 10.0% (standard deviation, SD = 2.53%) and 6.36% (SD = 2.32%), respectively (Figure 1). The time required for colonization with HLKR isolates was not significantly

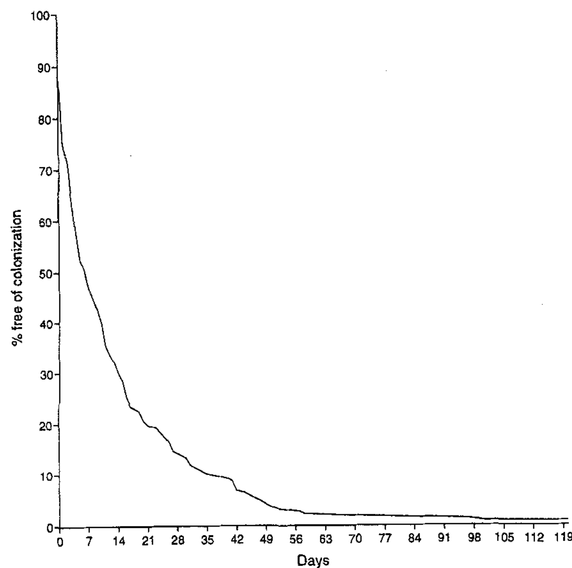

Figure 1 Estimation of the time required for colonization/infection with *E. faecalis* for the 217 colonized patients.

different to the time required for colonization with susceptible isolates ($p = 0.67$). Similarly, the times required for colonization with HLKR 'unique' pattern isolates and HLKR 'epidemic' pattern isolates were not significantly different (p=0.48). The time required for colonization/infection with HLGR isolates reached borderline statistical significance (25.33 days for colonization with HLGR isolates versus 13.15 days for susceptible isolates, p=0.069).

Rates of resistance

In total, 183 (81.3%) *E. faecalis* isolates expressed one or several mechanisms of acquired resistance: 106 (47.1%) isolates were highly resistant to kanamycin; 16 (7.1%) isolates were highly resistant to gentamicin; none was resistant to glycopeptides, and none produced a β-lactamase. Overall resistance rates were: erythromycin 73.8% (166 isolates), chloramphenicol 47.1% (106 isolates), and tetracycline 72.9% (164 isolates). Resistance to erythromycin, chloramphenicol and tetracycline was more frequent among isolates with high-level resistance to aminoglycosides (Table 2). Eleven (68.7%) isolates with HLGR and 74 (69.8%) isolates with HLKR were resistant to all antibiotics tested, except vancomycin and potent anti-*E. faecalis* β-lactams.

Molecular epidemiology

The 209 isolates from patients hospitalized in Besançon hospital or in other hospitals in eastern France yielded 53 major DNA patterns among susceptible isolates, 15 major DNA patterns among HLGR isolates, and 40 major DNA patterns among HLKR isolates (Table 3). Some patterns included isolates with different antibiotic phenotypes, so the total number of major DNA patterns was 98. One major epidemic pattern included 39 isolates: 28 isolates from periods A and B (22 of the 66 isolates from patients in Besançon hospital and six of the 30 isolates from patients hospitalized in the other hospitals in eastern France), nine of the 53 isolates from patients hospitalized in 1994 before the two study periods, and two of the 10 isolates provided by the community laboratories. Among these 39 isolates, six were susceptible isolates, two were HLGR isolates, and 31 were HLKR isolates (Figures 2 and 3). A second major epidemic pattern included 32 isolates, of which 16 were isolated during the study periods (11 isolates from Besançon hospital and five isolates from other hospitals), 14 were isolated in 1994 before the study periods, and two were provided by the community laboratories. Among these 32 isolates, two were susceptible isolates, two were HLGR isolates, and 28 were HLKR isolates. Thirty-seven HLGR isolates were typed and yielded 29 different DNA patterns, of which none corresponded to an epidemic strain.

Clinical epidemiology

The characteristics of the patient populations included in the two case-control studies are given in Table 4. After univariate analysis, the one variable significantly associated with HLGR colonization was the duration of hospitalization before colonization (p=0.043). No other factors were significantly associated with colon-

Table 2 Rates of co-resistance among HLGR and HLKR isolates

	High-level resistance to gentamicin			High-level resistance to kanamycin		
	+	−	RR (p)	+	−	RR (p)
Erythromycin	15	151	1.29 (0.07)	98	53	1.78 ($<10^{-5}$)
Chloramphenicol	11	95	1.51 (0.07)	76	19	3.85 ($<10^{-5}$)
Tetracycline	16	148	1.41 (0.007)	105	43	2.35 ($<10^{-5}$)

RR= relative risk

Table 3 Association of DNA patterns with different antibiotic susceptibility phenotypes

	Susceptible phenotype *n* (%)	HLGR phenotype *n* (%)	RR CI 95%	Susceptible phenotype *n* (%)	HLKR phenotype *n* (%)	RR CI 95%
Isolates	90	23	–	90	96	–
Major patterns	53	15	–	53	40	–
UP isolates	36 (40)	11 (47.8)	1.20			0.81
			0.73–1.96	36 (40)	29 (30.2)	0.59–1.10
EP isolates	16 (17.7)	7 (30.4)	1.71	16 (17.7)	55 (57.3)	3.22
			0.80–3.66			2.0–5.2

UP = unique pattern; EP = epidemic pattern; RR = relative risk

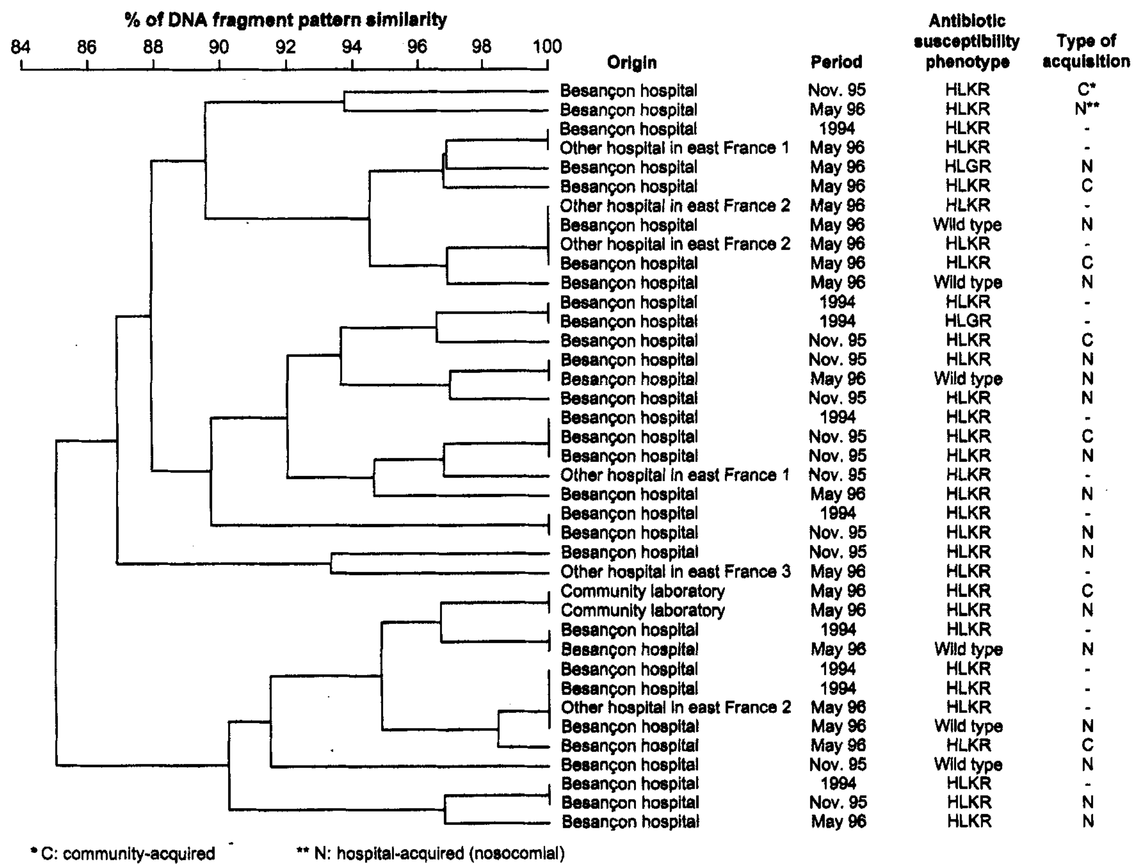

Figure 2 DNA fragment pattern similarity and epidemiologic information for *E. faecalis* isolates belonging to the first major epidemic pattern (DNA similarity coefficient of ≥85%.

ization or infection with either HLGR isolates or HLKR isolates (Table 5). The same variables were also analyzed for colonization/infection with HLKR isolates with the epidemic DNA pattern (n=17) versus HLKR isolates with a unique DNA pattern (n=12), but no single factor was significantly associated.

DISCUSSION

This study confirms the results of previous studies showing that *E. faecalis* colonizes/infects a large number of hospitalized patients in a wide variety of sites, but predominantly the urinary tract [27–30]. Many authors

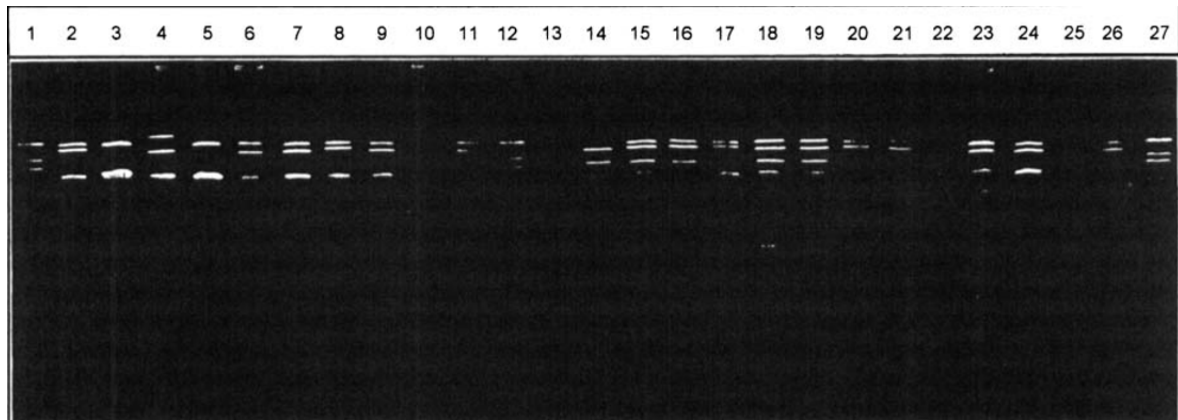

Figure 3 PFGE profiles of *Sma*I-digested DNA from *E. faecalis* isolates. Lanes 1, 12 and 27: *Staphylococcus aureus* NCTC 8325 DNA. Lanes 2–11 and 13–26: variants of one major epidemic pattern comprising 39 isolates.

Table 4 Characteristics of cases and controls

	No. (%)±SD		
Variable	HLGR *E. faecalis*, *n*=15	HLKR *E. faecalis*, *n*=110	Controls, *n*=86
Male	5 (53)	56 (51)	40 (47)
Mean age (years)	62.5±25.1	50.7±24.5	54.2±25.6
Number of hospitalization units	7	26	24
Previous hospitalization in another unit	5 (33)	28 (25)	22 (26)
Hospital service			
Medicine	11 (73)	67 (61)	46 (53)
Surgery	4 (27)	43 (39)	40 (47)
Type of acquisition			
Community	2 (13)	26 (24)	23 (27)
Anatomic site of isolation			
Urine	5 (33)	65 (59)	61 (71)
Superficial swabs	4 (27)	20 (18)	7 (8)
Wound	0 (0)	3 (3)	4 (5)
Blood	0 (0)	0 (0)	3 (3)
Other	6 (40)	22 (20)	11 (13)
Mean of days of hospitalization before colonization/infection	25.3±28.4	12.5±16.1	13.1±17.2
Antimicrobial therapy			
Any antibiotic	2 (13)	27 (25)	22 (26)
Potent antibiotic[a]	0 (0)	15 (14)	16 (19)
Less potent antibiotic[b]	2 (13)	15 (14)	10 (12)

[a and b] See Materials and Methods for listing of these antibiotics.

Table 5 Univariate analysis of risk factors

	HLGR cases versus controls		HLKR cases versus controls	
Risk factor	Odds ratio	CI 95%	Odds ratio	CI 95%
Male	0.76	0.25–2.32	0.90	0.51–1.60
Age (years)	1.01	0.99–1.04	0.99	0.98–1.01
Previous hospitalization in another unit	1.45	0.44–4.79	0.99	0.52–1.90
Hospitalization in surgery unit	0.42	0.12–1.44	0.74	0.41–1.31
Days of hospitalization before colonization	1.03[a]	1.00–1.05	1.00	0.99–1.01
Antimicrobial therapy	0.45	0.092–2.18	0.95	0.49–1.82
Potent antibiotic	NE	–	0.72	0.33–1.58
Less potent antibiotic	0.98	0.19–5.15	1.16	0.48–2.76

[a]$p=0.043$. NE, not evaluable.

have studied nosocomial enterococcal infections. However, the incidence in relation to the length of hospital exposure has not been investigated by Kaplan–Meier estimates or instantaneous risk calculations. The time required for colonization/infection is important for the choice of empirical therapy. The instantaneous risk suggests that the rate of colonization/infection with *E. faecalis* decreases with length of hospital exposure (0.0935 within the first week versus 0.0621 within the third week) and Kaplan–Meier estimates support this observation (4-day and 7-day rates were 10.0% and 6.36%, respectively). Although enterococci are now firmly established as major nosocomial pathogens, nearly 25% of *E. faecalis* isolates from our hospitalized patients were community acquired [31]. Among episodes of *E. faecalis* hospital-acquired colonization/infection, 22.8%, 10.6% and 66.6%, respectively, were classified as early, intermediate and late onset, with days 4 and 7 being the breakpoints (Figure 1).

Large numbers of *E. faecalis* isolates in Besançon hospital acquired resistance to aminoglycosides, of which nearly 50% possessed high-level resistance to kanamycin and 7% carried high-level resistance to gentamicin, consistent with the distribution of aminoglycoside resistance worldwide [32]. The synergistic activity of the combination of a penicillin with an aminoglycoside is abolished even when the aminoglycoside is a poor enzyme substrate and its bacteriostatic activity is not significantly affected. A high incidence of high-level resistance to aminoglycosides, whatever the enzyme, is known to affect current treatment of such infections [33]. The time required for colonization/infection with HLKR strains was not significantly different from that with susceptible strains, and the rate of HLKR among hospital-acquired isolates was not significantly different from that among community-acquired isolates. Thus, neither the time to occurrence nor the type of acquisition need be taken into account when choosing the treatment for severe infection where combination therapy is necessary. Most *E. faecalis* isolates are susceptible to ampicillin and glycopeptides [34]. Ampicillin resistance caused by the production of β-lactamase or a modified penicillin-binding protein has been reported for *E. faecalis* and *E. faecium*, respectively [32], but β-lactamase production was not detected in any of the *E. faecalis* isolates from Besançon. The ability of enterococci to acquire new resistance determinants is extended to antibiotics that are not used to treat enterococcal infections because of their weak activity against these organisms [33]. Most strains in Besançon were also resistant to tetracyclines, macrolides and chloramphenicol.

In eastern France, clonal spread makes a large contribution to the high prevalence of HLKR *E. faecalis* isolates. Thus, isolates with identical PFGE patterns were found in different hospitals, suggesting inter-hospital transmission. However, there is evidence of clonal dissemination of some epidemic strains in the community: (1) the prevalence of HLKR strains among community-acquired isolates was not lower than that among nosocomial isolates and (2) some isolates with major epidemic DNA patterns were from community laboratories. It should be noted, however, that the classification of cases as community acquired and the isolation of the strains in community laboratories does not exclude the possibility that patients may have acquired HLKR enterococci during a previous hospital admission. The observation that a single strain type was able to emerge among multiple other resistant strains to become the dominant HLKR *E. faecalis* strain in hospitals (independently of the different possible risk factors studied) suggests that this strain may have characteristics that differ from those of other equally resistant strains that allow it to persist and spread. The DNA heterogeneity among HLGR *E. faecalis* isolates suggests that clonal dissemination is not responsible for the spread of these resistant strains and confirms the previous studies of Zervos et al [35] and Thal et al [14].

The observed association between use of antibiotics and colonization/infection with HLGR *E. faecalis* is supported by several previous studies, including cephalosporins and aminoglycosides in the study by Zervos et al [35] on 96 patients infected in diverse sites, and cephalosporin in the studies by Noskin et al [36] and Huycke et al [37] for bloodstream infections. The present study on HLKR and HLGR isolates, as with the study of Antalek et al [12] of HLGR bloodstream isolates, did not identify previous antimicrobial therapy as a risk factor. In our series, the small number of HLGR cases may be responsible for the failure to identify a correlation, but there was also no correlation between the large number of HLKR cases and antimicrobial therapy. Another possible explanation for the difference between studies was the length of observed antimicrobial chemotherapy, which was not specified in some studies [12,36], but ranged from 1 week in the present study to 3 months before *Enterococcus* isolation [35], and also varied according to the length of hospitalization [36]. In our study, as in the study of Zervos et al [35], HLGR cases and controls had similar demographic characteristics. Other risk factors for HLGR *E. faecalis* colonization/infection identified in different studies are surgical procedures and length of hospitalization before isolation [35]. In the study of Antalek et al [12], none of these factors was significantly associated with colonization by HLGR enterococci. In the present study, the length of hospitalization before colonization with HLGR isolates

reached borderline significance in univariate analysis ($p=0.043$), and the time required for colonization with HLGR isolates reached borderline significance in the log-rank test ($p=0.069$).

Woodford et al [38] demonstrated linkage of the vancomycin resistance and high-level gentamicin resistance genes on the same plasmid in a clinical isolate of *E. faecalis*, the presence of the gentamicin resistance gene on a variety of physically distinct conjugative and non-conjugative plasmids in *E. faecalis* [35], and the wide dissemination of multidrug-resistant strains with characteristics that allow them to persist and spread. These observations, consistent with our study, indicate that it would be valuable to perform further large prospective surveys of clinical isolates of *E. faecalis* in hospitals, combined with studies of possible community dissemination of resistant strains.

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