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Emergence of Clonal Complex 17 *Enterococcus faecium* in The Netherlands▽

Janetta Top,[1]* Rob Willems,[1] Saskia van der Velden,[1] Miranda Asbroek,[1] and Marc Bonten[1,2]

Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands,[1] and Julius Center for Health Studies and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands[2]

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The global emergence of vancomycin-resistant *Enterococcus faecium* has been characterized as the clonal spread of clonal complex 17 (CC17) *E. faecium*. CC17 was defined upon multilocus sequence typing and is characterized by resistance to quinolones and ampicillin and the presence of the enterococcal surface protein (Esp) in the majority of isolates. The recently noticed increased incidence of vancomycin-susceptible CC17 *E. faecium* infections in our hospital initiated a nationwide study to determine ecological changes among enterococcal infections. The data and strain collections were obtained from 26 (38%) and 9 (14%) of 66 microbiology laboratories in The Netherlands. *E. faecium* and *E. faecalis* were distinguished by multiplex PCR; all *E. faecium* isolates were genotyped by multiple-locus variable-number tandem-repeat analysis (MLVA), and the presence of *esp* was identified by PCR. Average numbers of ampicillin-resistant enterococcal isolates from normally sterile body sites per hospital increased from 5 ± 1 in 1994 to 25 ± 21 in 2005. Among all enterococcal bloodstream infections, the proportions of ampicillin-resistant *E. faecium* (AREF) increased from 4% in 1994 to 20% in 2005 ($P < 0.001$). All *E. faecalis* isolates were susceptible to ampicillin, whereas 78% of the *E. faecium* isolates were resistant (49% of these contained *esp*). Genotyping revealed that 86% of AREF isolates belonged to CC17, including four dominant MLVA types found in ≥3 hospitals, accounting for 64% of the AREF isolates. Infections caused by CC17 *E. faecium* has increased nationwide, especially in university hospitals due to the clonal spread of four MLVA types, and seems associated with acquisition of the *esp* gene.

The emergence of vancomycin-resistant *Enterococcus faecium* (VREF) in the United States in the 1990s was preceded by the emergence of ampicillin-resistant *Enterococcus faecium* (AREF) in the 1980s (8, 11, 27, 28). Molecular epidemiological studies of human- and animal-derived *E. faecium* since then, revealed the existence of a genetic lineage, labeled clonal complex 17 (CC17), associated with nosocomial *E. faecium* outbreaks and infections in five continents. CC17 is characterized by ampicillin and quinolone resistance and the presence of a putative pathogenicity island, including the *esp* gene in the majority of isolates (2–4, 9, 12, 17–20, 31, 36). In retrospect, it seems likely that the acquisition of ampicillin resistance was an earlier step in hospital adaptation of *E. faecium*, facilitating the subsequent emergence of VREF (18, 36).

Since 2000, infection rates of VREF are rising in European hospitals (see EARSS Annual Report 2005 [www.rivm.nl/earss]), suggesting that the increase of VREF in Europe follows the American epidemiology with a 10-year delay. Little is known, though, about the molecular epidemiology of AREF.

In our hospital (the University Medical Center Utrecht [UMCU]), the proportion of invasive enterococcal infections caused by AREF increased from 2% in 1994 to 32% in 2005, with partial replacement of ampicillin-susceptible (Amp^s) *E. faecalis* by *E. faecium* (75% AREF) among enterococcal bloodstream infections (32). Based on these local findings, a nationwide study was initiated to determine the ecological changes among enterococcal infections from sterile body sites in hospitals in The Netherlands.

MATERIALS AND METHODS

Microbiology data. All microbiology laboratories ($n = 66$) serving 9 university and 87 nonuniversity hospitals in The Netherlands were invited to submit data on annual numbers of ampicillin-resistant (Amp^r) enterococci isolated from normally sterile body sites identified between 1994 and 2005. Normally sterile body sites included blood, abdominal and cerebrospinal fluid, intravascular catheter tips, and pus and wound specimens. These data did not differentiate enterococci to the species level.

Furthermore, the laboratories were invited to provide, for each year, the first 30 enterococcal bloodstream isolates, irrespective of antibiotic susceptibility (1 per patient). A species-specific multiplex PCR based on the *ddl* gene was performed to distinguish *E. faecium* and *E. faecalis* as previously described (6, 32). Susceptibilities to ampicillin were determined by inoculation of Mueller-Hinton agar containing ampicillin at 16 mg/liter according to Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) guidelines.

Genotyping of *E. faecium* isolates. All *E. faecium* isolates, including 2006 isolates, were genotyped by using multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA), as described previously (31) with minor modifications (www.mlva.umcutrecht.nl). Identification of CC17-specific MLVA types (MTs) was performed by comparing each MLVA profile to the previously described seven different repeat combinations for VNTR-7, -8, and -10 with a positive predictive value of 87% and a specificity of 90% to belong to CC17 (31). The genetic relatedness of MTs was confirmed by multilocus sequence typing (MLST) on a subset of representative isolates (9). The obtained MLST profiles were clustered with 313 MLST profiles, representing 855 isolates from the database using the eBURST algorithm (7, 18). The presence of the putative pathogenicity island was determined by PCR using the *esp* gene as a marker (20).

Statistical analysis. Statistical analysis of the data was performed with SPSS 12.0.1 for Windows (SPSS, Inc., Chicago, IL) using the chi-square test. The data from university hospitals were compared to those from nonuniversity hospitals.

RESULTS

Microbiology data invasive Amp^r enterococci. Of 66 microbiology laboratories serving 7 of 9 (78%) university hospitals

* Corresponding author. Mailing address: Department of Medical Microbiology, University Medical Center Utrecht, G04.614, P.O. Box 85500, 3508 GA Utrecht, The Netherlands. Phone: 31-88-7557627. Fax: 31-30-2541770. E-mail: j.top@umcutrecht.nl.

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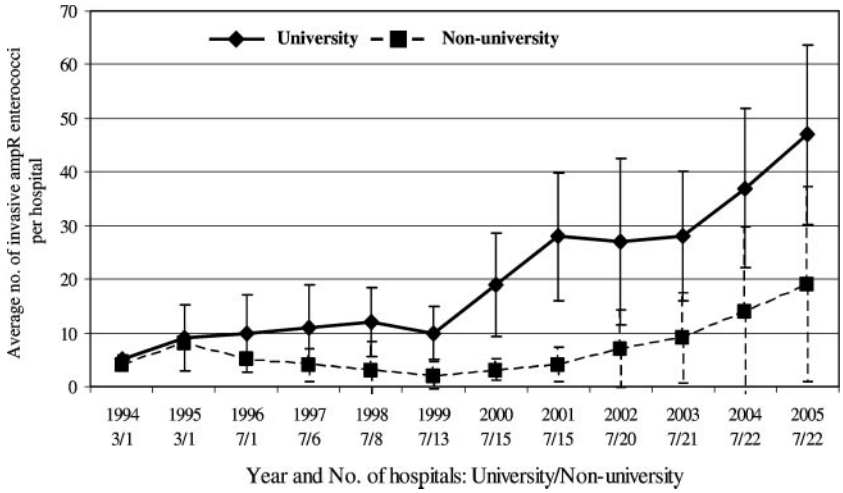

FIG. 1. Average annual numbers of invasive Amp^r enterococci per hospital. Error bars denote standard deviations. University and nonuniversity hospitals were compared. For each year, the numbers of hospitals that provided data are indicated.

(>500 beds) and 22 of 87 (25%) nonuniversity hospitals (250 to 500 beds [$n = 6$], >500 beds [$n = 16$]), 26 (39%) provided data on Amp^r enterococci from normally sterile body sites. The data from our own hospital, already described previously (32), were included as well. The hospitals were dispersed throughout The Netherlands. Only one nonuniversity and three university hospitals could provide data going back as far as 1994.

Average annual numbers of Amp^r enterococci from normally sterile body sites per hospital increased from 5 ± 1 in 1994 to 25 ± 21 in 2005. The increase was most pronounced in university hospitals (from 5 ± 1 in 1994 to 47 ± 17 in 2005) (Fig. 1). The average annual numbers in nonuniversity hospitals increased from 4 ± 0 in 1994 to 19 ± 18 in 2005 (Fig. 1). Annual numbers per hospital varied between 1 and 14 for 250- to 500-bed hospitals and between 1 and 80 for larger hospitals (>500 beds).

***E. faecium*/*E. faecalis* ratio among bloodstream isolates.** In all, 1,573 enterococcal bloodstream isolates were obtained from nine hospitals (five nonuniversity and four university). Three of the four university hospitals provided isolates from 1994 onward. The oldest isolates obtained from a nonuniversity hospital were from 1999.

Species identification revealed 1,121 *E. faecalis*, 303 *E. faecium*, and 149 non-*E. faecalis* and non-*E. faecium* isolates. The latter isolates were not further characterized. Discrepancies between the original identification, as provided by the submitting labs and identification based on the *ddl* gene, were found in 116 (7%) isolates. All *E. faecalis* isolates were Amp^s, whereas 237 of 303 (78%) *E. faecium* isolates were Amp^r.

The proportions of AREF among all enterococcal bloodstream isolates increased from 4% (1994) to 20% (2005) ($P = 0.01$), while the proportions of Amp^s *E. faecalis* decreased from 89% (1994) to 77% (2005) ($P = 0.5$). Proportions of Amp^s *E. faecium* remained <9%, and no significant trend could be observed over time. In university hospitals the proportions of AREF increased from 4% in 1994 to 27% in 2005 ($P < 0.001$). For individual hospitals these proportions ranged from 0% in 1994 to 10% in 2005 (lowest) and from 27% in 1996 to 43% in 2005 (highest). In nonuniversity hospitals there was a slight, but nonsignificant increase in the proportions of AREF from 6% in 1999 to 12% in 2005.

Genotyping *E. faecium* isolates. MLVA typing of 303 *E. faecium* isolates revealed 61 different MTs among 263 isolates, including 41 MTs that had not been previously detected. Incomplete MLVA profiles were obtained for 29 isolates due to repeatedly negative PCR results for ≥1 of the VNTR loci. In 11 isolates none of the VNTR loci were PCR positive. All 40 isolates that could not be assigned a MT appeared to be Amp^s. Twenty of the remaining 26 (77%) Amp^s *E. faecium* isolates yielded a unique MT.

Sixty-seven percent (175 of 263) of typeable isolates belonged to five MTs, including four MTs detected in ≥3 hospitals: MT-1 ($n = 97$ in 9 hospitals) MT-5 ($n = 19$ in 5 hospitals), MT-12 ($n = 18$ in 6 hospitals), and MT-159 ($n = 17$ in 5 hospitals), together accounting for 64% (151 of 237) of Amp^r isolates (Table 1). MT-22 (24 of 303 isolates) was detected in only one hospital, where it accounted for 29% (24 of 83) of all *E. faecium* isolates between 1999 and 2003 (Fig. 2).

Longitudinal analysis of the genotyping data revealed that MT-1 was already present in one hospital in 1994 and that its presence increased after 1999, with a documented presence in all nine hospitals (Fig. 2 and Table 1). MT-5 and MT-12 emerged from 1999 and 2002 onward (Fig. 2). The first MT-12 isolate was detected in one hospital in 2002, and it appeared in three other hospitals in 2006 (Table 1). Finally, MT-159 was found in two hospitals in 2005, with subsequent isolation in three additional hospitals in 2006.

The four most predominant MTs detected in ≥3 hospitals were closely related. MT-5 and MT-12 were single-locus variants from MT-1, while MT-159 was a double-locus variant from MT-1 and a single-locus variant from MT-12 (Table 1). Identification of CC17-specific MTs based on different repeat combinations for VNTR-7, -8, and -10 (31) revealed that 86% (204 of 237) of Amp^r isolates belonged to CC17 (Table 1).

MLST was performed on 15 Amp^r and 12 Amp^s isolates (Table 2). The seven MT-159 isolates of different hospitals revealed a single sequence type (ST), ST-78. In contrast, seven MT-12 isolates from different years represented five different

TABLE 1. Distribution of predominant MTs

MT	MLVA profile (no. of repeats)						CC17-specific MTs[a]	Total no. of isolates	No. of Amp^r isolates	No. of *esp*-positive isolates	No. of hospitals
	VNTR-1	VNTR-2	VNTR-7	VNTR-8	VNTR-9	VNTR-10					
1	5	7	3	3	2	3	+	97	97	19	9
5	5	7	3	2	2	3	+	19	19	17	5
12	5	7	3	3	1	3	+	18	18	16	6
22	5	7	4	2	2	1	–	24	24	24	1
159	5	7	3	3	1	2	+	17	17	17	5

[a] +, MTs with the following repeat profiles for VNTR-7, -8, and -10: 3-3-3, 3-2-3, 3-3-2, 4-3-3, 3-4-3, 4-2-3, and 3-3-1.

STs. All STs representing Amp^r isolates, except one (ST-324), grouped within or were linked to CC17 (Fig. 3). The Amp^s *E. faecium* isolates, all MLVA nontypeable, revealed different STs, including seven new STs, ST-326 to -332, ST-334, and ST-100, -52, -272, and -296. Six STs clustered with other ampicillin- and vancomycin-susceptible human community isolates, including MLVA nontypeable *E. faecium* isolates, four represented singletons, and one isolate grouped among poultry isolates, and one ST (ST-326) was linked to CC17 (Fig. 3).

Determination of *esp* gene. Forty-nine percent (115 of 237) of Amp^r *E. faecium* isolates contained the *esp* gene, while none of the Amp^s isolates was *esp* positive. In longitudinal analysis a remarkable increase of *esp*-positive isolates occurred from 2004 onward (Fig. 4). The total numbers of *esp*-negative isolates peaked in 2003 (n = 40) and decreased subsequently. Interestingly, all MT-12 isolates from 2002 and 2003 were *esp* negative, whereas all MT-12 isolates from 2005 onward contained the *esp* gene. Similarly, the majority of *esp*-positive isolates among MT-1 isolates (15 of 19 [79%]) were found between 2004 and 2006. Before 2004, only 4 of 47 MT-1 isolates (9%) were *esp* positive. Finally, 17 of 19 MT-5 isolates (89%) and all MT-159 isolates were *esp* positive. These findings suggest that MT-1 and MT-12 isolates acquired the *esp* gene and that the presence of this gene was associated with nosocomial spread. On the other hand, *esp*-positive MT-22 isolates were only found in one hospital and apparently disappeared in 2003, and MT-5 *esp*-positive isolates were detected in low numbers in five hospitals, without evidence of increased nosocomial spread during the years (Table 1).

DISCUSSION

The present study demonstrates a nationwide increase of CC17 AREF isolates obtained from normally sterile body sites in The Netherlands. The molecular epidemiology is characterized by the emergence of several clones, with presumed intra- and interhospital spread. The presence of the *esp* gene, previously described as a marker of a putative pathogenicity island, seems strongly associated with the emergence of CC17 AREF. The partial replacement of Amp^s *E. faecalis* by CC17 AREF has consequences for antimicrobial treatment of enterococcal infections and, more importantly, may set the stage for the emergence of vancomycin-resistant *E. faecium*.

Our study was based on the voluntary collaboration of microbiological laboratories in The Netherlands and therefore has some potential limitations. In all, 39% of all laboratories provided information on annual numbers of Amp^r enterococci obtained from normally sterile body sites. Eight laboratories did not have computerized data information, and thirty hospitals never responded to our (once-repeated) request. Since we failed to obtain information from all laboratories, some selection bias cannot be fully excluded. However, only one of the

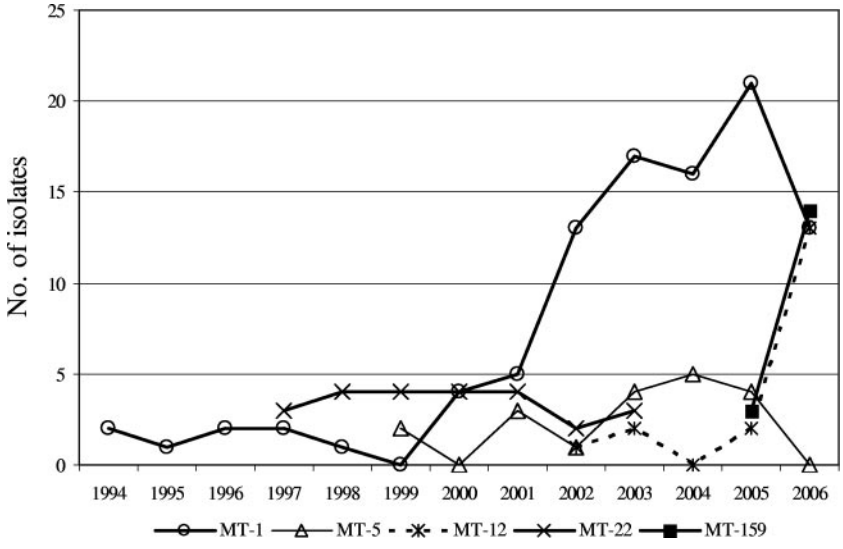

FIG. 2. Annual distribution of five predominant MTs.

TABLE 2. MLST results for representative MTs

Resistance	MT (no. of isolates typed)	No. of hospitals	*esp* gene	Yr	MLST
Amp^r	MT-159 (7)	5	+	2005/2006	ST-78
	MT-12 (3)	3	–	2002/2003	ST-18, ST-324, ST-325
	MT-12 (1)	1	+	2005	ST-78
	MT-12 (3)	3	+	2005/2006	ST-117
	MT-22 (1)	1	+	2000	ST-16
Amp^s	Nontypeable (12)	8	–	NA[a]	New: ST-326 to ST-332, ST-334; ST-100, ST-52, ST-272, ST-296

[a] NA, not applicable.

participating hospitals (large nonuniversity hospital) had identified a nosocomial outbreak with AREF before our study request. On the other hand, three hospitals that did not participate reported the emergence of AREF infections (all MT-159) in 2006 (unpublished data).

Furthermore, one of the participating nonuniversity laboratories, representing 4.4% of the total nonuniversity isolates and 1.9% of the total number of isolates, could not provide information on isolation sites and, therefore, some isolates might not reflect invasive infections. However, this would ac-

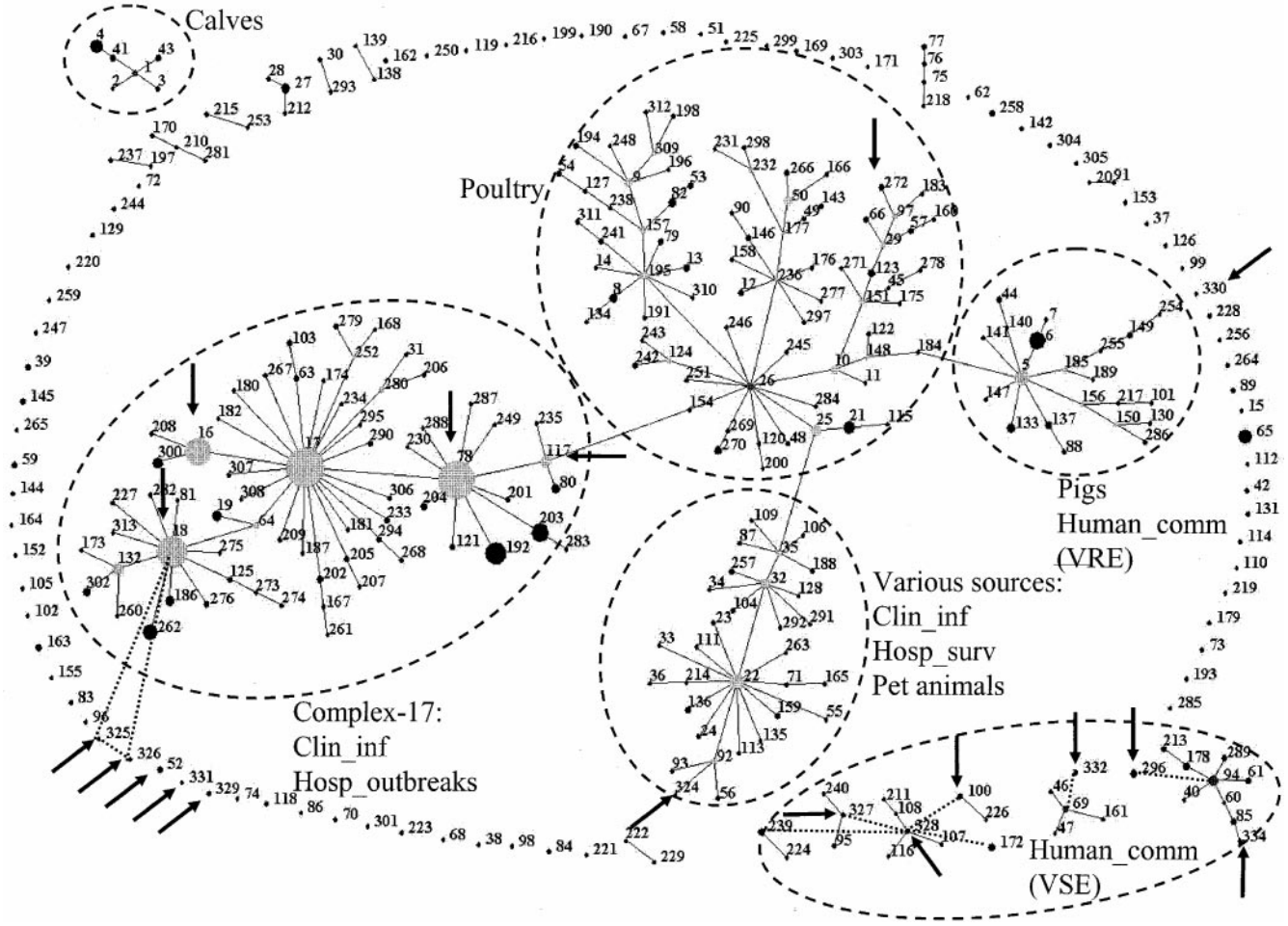

FIG. 3. eBURST clustering of 18 MLST profiles, indicated by an arrow, representing 27 isolates from present study, with 313 MLST profiles representing 855 *E. faecium* isolates from the database (www.mlst.net). Each ST is represented as a node; the relative size of each node is indicative of its prevalence among the isolates, and lines connect single-locus variants (STs that differ in only one of the seven housekeeping genes). Dashed lines indicate connections between double-locus variants. The sources of specific clusters of STs are indicated, including CC17 comprising hospital outbreaks and clinical isolates. Clin_inf, isolates from clinical sites (mainly blood) from hospitalized patients; Hosp_outbreak, hospital outbreak isolates; Hosp_surv, feces isolates from hospitalized patients without an enterococcal infection and not associated with an enterococcal hospital outbreak; Human_comm, feces isolates from human volunteers not connected to hospitals.

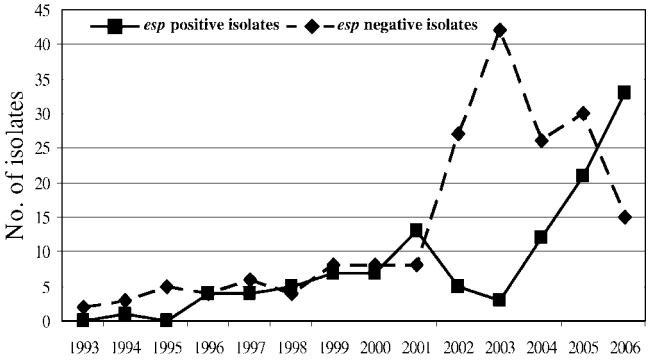

FIG. 4. Comparison of the annual distribution of *esp*-positive and -negative isolates.

count only for urine isolates, since surveillance for asymptomatic carriage with Amp^r enterococci had not been performed in any hospital. Few laboratories had stored enterococcal isolates, and nine could provide enterococcal bloodstream isolates. It is highly unlikely that hospitals preferably stored either *E. faecalis* or *E. faecium* isolates and, therefore, the reported proportions of AREF probably reflect an unbiased estimate. For all of these reasons, we consider the present study to be a reliable reflection of the enterococcal epidemiology in The Netherlands.

The increase and replacement of AREF was most pronounced in university hospitals and large nonuniversity hospitals (>500 beds), a finding that probably reflects differences in patient populations, compared to smaller hospitals. Hematology and transplant patients are generally considered at highest risk for enterococcal bacteremia (5, 32). In our hospital, the increase in AREF bloodstream infections was associated with increased fecal carriage of AREF among hospitalized patients (32). Point-prevalence studies revealed intestinal colonization with AREF in up to 35% of hospitalized patients, especially among high-risk patients on hematology and nephrology wards. Although colonization data are absent for other centers, the endemicity of intestinal colonization with AREF has probably been established in multiple hospitals in The Netherlands.

MLVA typing revealed four highly related types to be responsible for the nationwide emergence of AREF. MT-159 *E. faecium* isolates first appeared in one hospital in 2005, with documented presence in five hospitals in 2006. However, outbreaks of AREF documented in three other hospitals and not included in the present study were also caused by MT-159 isolates (data not shown). MLST of representative MT-159 isolates (also from the three hospitals not included in the study) revealed ST-78. Nosocomial outbreaks of ST-78 have been described in Korea and Europe, including Germany and Italy (1, 14, 16, 24).

Interestingly, the majority (57%) of *esp*-positive isolates were found from 2004 onward, and this gene was contained in MT-1 and its genetically related variants MT-12 and -159. We consider the *esp* gene to be a marker of a putative pathogenicity island (17). This sudden increase of *esp*-positive isolates suggests that MT-1 and MT-12 acquired the putative pathogenicity island via conjugative transfer, as has been shown in vitro (23), which might contribute to an increased ability to spread and cause infections. In a recent study, Esp expression on the surface of *E. faecium* varied substantially between isolates and was correlated with initial adherence to polystyrene and biofilm formation (35). Therefore, a role for Esp in the early stages of colonization and subsequent infection has been hypothesized (35).

Previously, MLST of several MT-1 isolates indicated that MT-1 is comprised of multiple STs, including ST-17, the presumed founder of CC17, thus representing a polyclonal population (31, 36). The observation that particular MTs, such as the MT-12 isolates from the present study, are represented by different types determined by MLST and vice versa has been reported before (31), and probably results from differences in the frequency in occurrence of changes in repeat numbers compared to DNA polymorphisms, mutation, and recombination in housekeeping genes.

MLVA typing of 40 Amp^s *E. faecium* isolates revealed incomplete MLVA profiles. Southern blot hybridization of three representative isolates confirmed the absence of at least one of the VNTR regions (data not shown). MLST of 12 MLVA nontypeable isolates confirmed that the Amp^s *E. faecium* isolates are not linked to CC17 but clustered with other MLVA nontypeable Amp^s *E. faecium* isolates that were not involved in hospital outbreaks.

In the United States, the emergence of AREF preceded the nationwide nosocomial epidemic of vancomycin-resistant enterococci. A changing *E. faecalis*/*E. faecium* ratio in hospital infections was reported in three longitudinal microbiology-based studies (10, 22, 34). In Europe, several reports on the increase in invasive AREF have been published (4, 29, 33), but to our knowledge ours is the first nationwide study in Europe on the molecular epidemiology of AREF. The emergence of CC17 AREF, resulting in changing *E. faecalis*/*E. faecium* ratios among bloodstream isolates, and with 78% of *E. faecium* isolated determined to be Amp^r will impact the treatment of enterococcal infections. The preferred antibiotic for invasive enterococcal infections, amoxicillin, must now be replaced by vancomycin, linezolid, or daptomycin. Increased use of these agents may create selective antibiotic pressure, facilitating the emergence of VREF due to the horizontal transfer of vancomycin resistance genes (13, 30, 32), to mutations leading to resistance to linezolid (15, 26, 37), or to an as-yet-undescribed resistance to daptomycin (21, 25).

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