Chapter 8

Emergence of Ampicillin-resistant CC17 *Enterococcus faecium* (AREfm) in the Netherlands

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Abstract

A nationwide study was performed to determine ecological changes among invasive enterococcal infections in the Netherlands. Twenty-six of 66 microbiology laboratory (38%, serving 29 hospitals) provided data, and 9 (14%) labs provided enterococcal isolates. Multiplex PCR based on the *ddl* gene was performed to distinguish *Enterococcus faecium* and *Enterococcus faecalis.* All *E. faecium* isolates were genotyped with multiple locus variable number tandem repeat analysis (MLVA) and representative isolates with multi-locus sequence typing (MLST). Finally, the presence of the putative pathogenicity island was determined by PCR using the *esp* gene as a marker. The average number of invasive ampicillin resistant (ampR) enterococcal isolates per hospital increased from 5 ± 1 in 1994 to 25 ± 21 in 2005, and was most pronounced in university hospitals. Proportions ampicillin resistant *E. faecium* (AREfm) among all enterococcal bloodstream isolates increased from 4% in 1994 to 20% in 2005 ($p<0.001$), again predominantly in university hospitals. All *E. faecalis* isolates were ampicillin susceptible, while 78% of the *E. faecium* isolates were ampicillin resistant. MLVA typing of all (n=303) *E. faecium* isolates revealed 61 MLVA types (MT). Four predominant types (MT-1, -5, -12 and -159) belonging to CC17 were found in ≥ 3 hospitals. Forty-nine percent of ampR isolates contained the putative pathogenicity island, while none of the ampS isolates were *esp* positive. A sudden increase of *esp* positive isolates was observed from 2004 on, including MT-1, -12 and -159 isolates. Invasive AREfm, belonging to CC17, have increased nationwide, especially in university hospitals and have partially replaced ampS *E. faecalis*. This rapid emergence has resulted from clonal spread of 4 MLVA types and seems associated with acquisition of the *esp* gene in two genotypes.

Introduction

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* (AREfm) in the 1980s (8,10,25,26). 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 (2-4,9,11,16-19,28,31).

Since 2000, infection rates of VREF are rising in European hospitals (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. In retrospect, it

seems likely that acquisition of ampicillin resistance was an earlier step in hospital adaptation of *E. faecium*, facilitating the subsequent emergence of VREF (17,31). Little is known, though, about the molecular epidemiology of AREfm.

In our hospital (the University Medical Center Utrecht (UMCU)), the proportion of invasive enterococcal infections caused by AREfm increased from 2% in 1994 to 32% in 2005 with partial replacement of ampicillin-susceptible (ampS) *E. faecalis* by *E. faecium* (75% AREfm) among enterococcal bloodstream infections (29). Based on these local findings, a nationwide study was initiated to determine the ecological changes in enterococcal populations in hospitals in the Netherlands.

Materials and methods

Microbiology data

All microbiology laboratories (n=66) serving hospitals in the Netherlands were invited to submit data on annual numbers of invasive ampicillin resistant (ampR) enterococcal infections identified between 1994 and 2005. Invasive infections were defined as infectious episodes with ampR enterococci isolated from normally sterile body sites like blood, abdominal – and cerebrospinal fluid, intravascular catheter tips, pus and wound specimens.

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 (6,29). Susceptibilities to ampicillin were determined by inoculation of Mueller-Hinton agar containing ampicillin 16 mg/L, according to CLSI (formerly NCCLS) guidelines.

Genotyping of *E. faecium* isolates

All *E. faecium* isolates were genotyped using multiple locus variable number tandem repeat analysis (MLVA), as described previously (28) with minor modifications (www.mlva.umcutrecht.nl). Identification of CC17 specific MLVA types was performed by comparing each MLVA profile with the previously described seven different repeat combinations for VNTR-7, -8 and -10 with a positive predictive value (PPV) of 87% and specificity of 90% to belong to CC17 (28). The genetic relatedness of MLVA types was confirmed with multi-locus 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 (7,17). The presence of the putative pathogenicity island was determined by PCR using the *esp* gene as a marker (19).

Statistical analysis

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

Results

Microbiology data invasive ampR enterococci

Twenty-six (39%) of 66 microbiology laboratories, serving 29 hospitals (seven university hospitals (> 500 beds) and 22 non-university hospitals (250-500 beds n=6, > 500 beds n=16), provided data on invasive ampR enterococcal isolates. The data from our own hospital, already described in the previous study (29), were included as well. The hospitals were dispersed over the Netherlands (Figure 1). Only one non-university and three university hospitals could provide data going back as far as 1994.

Average annual numbers of invasive ampR enterococcal isolates 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) (Figure 2). The average annual numbers in non-university hospitals increased from 4 ± 0 in 1994 to 19 ± 18 in 2005 (Figure 2). Annual numbers per hospital varied between 1 and 14 for 250-500-bed hospitals and between 1 and 80 for larger hospitals (>500 beds).

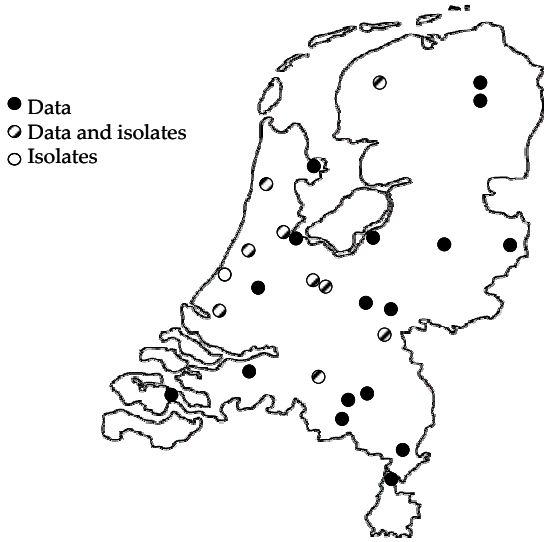

Figure 1. Distribution of contributing hospitals over the Netherlands. Solid dots represent hospitals, which provided data only; hatched dots hospitals, which provided data and bloodstream isolates and open dot, one hospital, which provided bloodstream isolates only

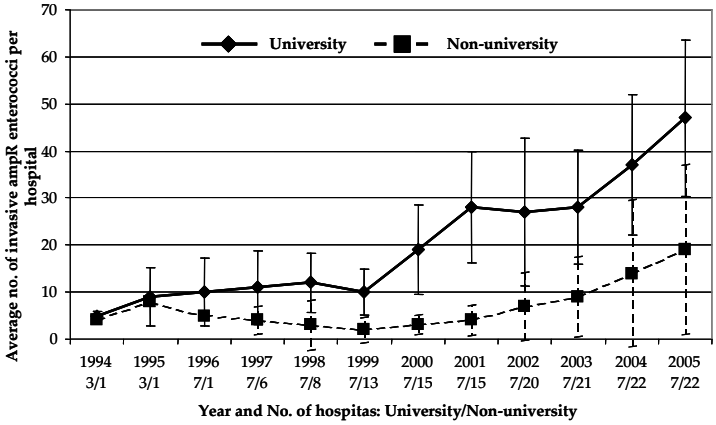

Figure 2. Average annual numbers of invasive ampR enterococci per hospital. Error bars denote standard deviations. Comparison of university- and non-university hospitals. For each year numbers of hospitals, which provided data are indicated.

E. faecium to *E. faecalis* ratio among bloodstream isolates

In all, 1573 enterococcal bloodstream isolates were obtained from 9 hospitals (5 non-university and 4 university). Three of the four university hospitals provided isolates from 1994 on. The oldest isolates obtained from a non-university hospital were from 1999.

Species identification revealed 1121 *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 susceptible to ampicillin, whereas 236 of 303 (78%) *E. faecium* isolates were ampicillin resistant.

Proportions of AREfm among all enterococcal bloodstream isolates increased from 4% (1994) to 20% (2005) (p =0.01), while proportions of ampS *E. faecalis* decreased from 89% (1994) to 77% (2005). Proportions of ampS *E. faecium* remained <12% and no significant trend could be observed over time. In university hospitals proportions AREfm increased from 4% in 1994 to 27% in 2005 (p <0.001) (Figure 3). 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 non-university hospitals there was a slight, but non-significant increase in proportions of AREfm from 6% in 1999 to 12% in 2005 (Figure 4).

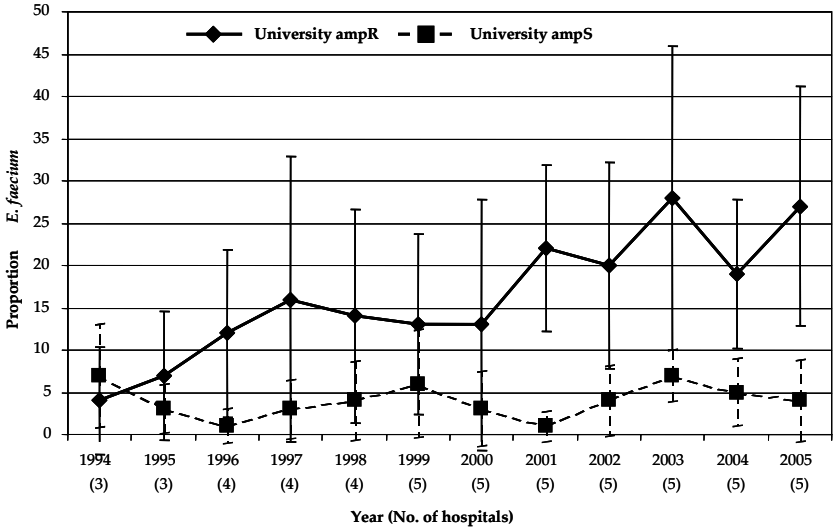

Figure 3. Average annual proportions ampicillin resistant (ampR) and ampicillin susceptible (ampS) *E. faecium* among enterococcal bloodstream isolates from university hospitals. Error bars denote standard deviations. For each year numbers of hospitals, which provided isolates are indicated.

Genotyping *E. faecium* isolates

MLVA typing of 303 *E. faecium* isolates revealed 61 different MLVA types (MTs) among 263 isolates, including 41 not previously found MTs (Table 1). Incomplete MLVA profiles were obtained for 30 isolates due to repeatedly negative PCR results for ≥ 1 of the VNTR loci. In ten isolates none of the VNTR loci were PCR-positive (Table 1). All 40 isolates that could not be assigned a MT, appeared to be ampicillin susceptible. Nineteen of the remaining 27 (70%) ampS *E. faecium* isolates yielded a unique MT.

Sixty-seven percent (175/263) of typable 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% (151/236) of ampR isolates (Table 1). MT-22 (24 of 303 isolates) was detected in only one hospital, where it accounted for 29% (24/82) of all *E. faecium* isolates between 1999 and 2003 (Figure 5).

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 documented presence in all 9 hospitals (Figure 5 and Table 1). MT-5 and MT-12 emerged from 1999 and 2002 on (Figure 5). 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 2 hospitals in 2005, with subsequent isolation in 3 additional hospitals in 2006.

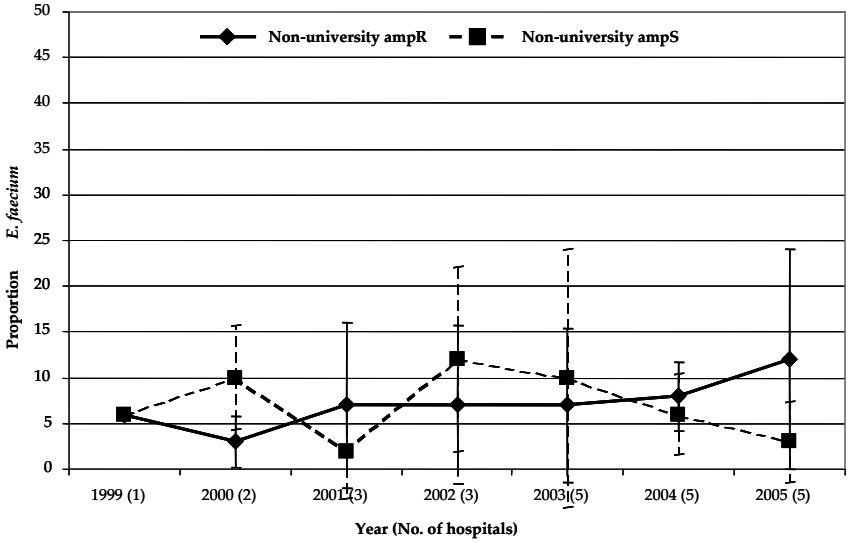

Figure 4. Average annual proportions ampicillin resistant (ampR) and ampicillin susceptible (ampS) *E. faecium* among enterococcal bloodstream isolates from non-university hospitals. Error bars denote standard deviations. For each year numbers of hospitals, which provided isolates are indicated.

The four most predominant MTs detected in ≥ 3 hospitals were closely related. MT-5 and MT-12 were single locus variants (SLV) from MT-1, while MT-159 was a double locus variant (DLV) from MT-1 and a SLV from MT-12 (Table 1). Identification of CC17 specific MLVA types based on different repeat combinations for VNTR-7, -8 and -10 previously shown to have a PPV of 87% and a specificity of 90% for CC17 isolates (28), revealed that 86% (204/236) of ampR isolates belonged to CC17, compared to 7% (2/27) of the ampS isolates (Table 1).

MLST typing was performed on MT-159 (n=7) and MT-12 (n=7) isolates of different hospitals and years (Table 2). All MT-159 isolates revealed a single sequence type (ST)-78. In contrast, seven MT-12 isolates had five different STs in MLST (Table 2). All STs representing ampR isolates, except one (ST-324) grouped within or were linked to CC17 (Figure 6). Ten-ampicillin susceptible *E. faecium* isolates, all MLVA non-typable, revealed different STs, including 7 new STs, ST-326 to -332 and ST-100, -52 and -272. Six STs clustered with other ampicillin- and vancomycin susceptible human community isolates, including MLVA non-typable *E. faecium* isolates, four represented singletons, one isolate grouped among poultry isolates and one ST (ST-326) was linked to CC17 (Figure 6).

Table 1. Distribution of MLVA types

	MLVA profile										
MLVA MT	VNTR-1	VNTR-2	VNTR-7	VNTR-8	VNTR-9	VNTR-10	CC17 specific MTs	No. of isolates	ampR	No.of esp positive isolates	No. of hospitals
1	5	7	3	3	2	3	+	**97**	97	19	9
2	5	6	3	3	2	3	+	3	3	3	2
3	7	7	3	3	2	3	+	6	6	1	3
4	5	4	3	3	2	3	+	4	4	1	2
5	5	7	3	2	2	3	+	**19**	19	17	5
7	5	7	3	3	2	2	+	1	1		1
8	5	7	4	3	2	3	+	2	2		1
12	5	7	3	3	1	3	+	**18**	18	16	6
13	5	7	3	4	3	3	+	4	4		2
14	7	7	3	4	3	3	+	1	1		1
16	5	6	3	2	2	3	+	5	5	5	3
22	5	7	4	2	2	1	-	24	24	24	1
30	6	4	3	3	3	3	+	1	1		1
31	6	4	3	3	3	1	+	2	2		2
39	4	7	1	3	2	3	-	3	3		2
50	5	7	3	4	1	3	+	1	1	1	1
54	5	7	5	4	1	3	-	1			1
57	6	7	2	4	1	3	-	1			1
89	6	4	3	1	1	1	-	1			1
95	5	2	3	2	1	1	-	1			1
139	6	6	3	3	1	3	+	1	1		1
144	5	7	3	3	3	2	+	2	2		1
152	5	7	3	4	2	3	+	1	1		1
159	5	7	3	3	1	2	+	**17**	17	17	5
164	11	7	3	3	2	3	+	3	3	3	1
205	4	7	3	3	2	3	+	1	1		1
214	5	6	3	2	3	3	+	2	2	2	2
226	4	7	1	4	1	3	-	1			1
228	6	7	4	4	1	3	-	2			2
230	5	7	3	3	2	1	+	1	1		1
237	5	7	4	2	1	3	+	3	3	3	1
253	4	5	6	3	1	3	-	1			1
254	4	7	6	4	2	3	-	1			1
255	5	6	3	4	2	4	-	1			1
256	5	6	3	2	1	3	+	1	1	1	1
257	5	6	7	6	3	3	-	2			1
258	5	7	3	2	1	3	+	1	1		1
259	5	7	3	6	1	3	-	2			1
260	5	7	4	2	1	1	-	1	1	1	1
261	6	4	7	3	1	2	-	1			1
262	6	6	2	4	2	3	-	1			1
263	6	6	3	3	3	3	+	2	2		2
264	6	6	3	4	1	3	+	1	1		1
265	6	7	5	4	2	3	-	1	1		1

Table 1. Distribution of MLVA types, continued

	MLVA profile										
MLVA MT	VNTR-1	VNTR-2	VNTR-7	VNTR-8	VNTR-9	VNTR-10	CC17 specific MTs	No. of isolates	ampR	No.of esp positive isolates	No. of hospitals
266	6	7	6	4	1	3	-	2			1
267	6	9	3	4	1	2	-	1	1	1	1
268	7	7	3	4	2	3	+	1	1		1
269	5	3	4	2	1	1	-	1			1
270	3	7	1	4	3	1	-	1			1
271	3	3	4	2	1	3	+	1	1		1
272	6	5	3	3	3	3	+	1	1		1
273	6	7	2	3	1	3	-	1			1
274	5	2	4	4	2	1	-	1		1	1
275	5	0	3	1	1	1	-	1			1
276	5	1	1	4	1	2	-	1			1
277	3	7	4	3	1	3	+	1			1
278	5	9	3	4	2	3	+	1			1
279	6	16	5	3	2	3	-	1			1
280	6	16	3	3	2	3	+	1	1		1
283	4	4	6	3	2	3	-	1	1		1
284	6	8	4	4	1	3	-	1	1		1
Incomplete MLVA profiles[a]	2		3		3	3		2			2
	2							1			1
	3	4		4	1	1		1			1
	3	6	2		2	2		1			1
	3							1			1
	3					3		1			1
	4	4	6	3	1			1			1
	4	7						1			1
	4		3	3	3	3		2			2
	4		3		3			1			1
	4							1			1
	4			3	2	3		1			1
	5	0		2	2			1			1
	5	1	3	1		2		1			1
	5	1		2	2	3		2			2
	5	7		2	2	3		1			1
	6	5	3	1		1		2			1
	6	5	3	3		3		1			1
	6	7	5	4		3		1			1
		7	3	3	1	3		1			1
			3	3				1			1
			3		3			1			1
				3	4	3		1			1
					2	3		1			1
						3		1			1
					2	3		1			1

[a] Of 10 ampS isolates represting 6 different hospitals none of the VNTR loci revealed a positve PCR result

[b] +; MTs with the following repeat profile for VNTR-7, -8 and -10: 3-3-3; 3-2-3; 3-3-2; 4-3-3; 3-4-3; 4-2-3; 3-3-1 were identified as belonging to CC17 with a sensitivity of 97% and a specificity of 90% (28)

Determination of *esp* gene

Forty-nine percent (116/236) of ampR *E. faecium* isolates contained the *esp* gene, while none of the ampS isolates was *esp* positive. In longitudinal analysis a remarkable increase of *esp* positive isolates occurred from 2004 on (Figure 7). 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 on contained the *esp* gene. Similarly, the majority of *esp* positive isolates among MT-1 isolates (15/19, 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 5 hospitals, without evidence of increased nosocomial spread during the years (Table 1).

Table 2. MLST typing results on representative MLVA types

Resistance	MLVA type (No. of isolates typed by MLST)	No. of hospitals	*esp* gene	year	MLST
ampR	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
ampS	non-typable (10)	8	-	n/a	new: ST-326 to ST-332
					ST-100, ST-52, ST-272

Discussion

The current study demonstrates a nationwide increase of invasive CC17 AREfm in the Netherlands. The molecular epidemiology is characterized by the emergence of several clones, with presumed intra- and inter-hospital spread. The presence of the *esp* gene, previously described as marker of a putative pathogenicity island, seems strongly associated with the emergence of CC17 AREfm. The partial replacement of ampS *E. faecalis* by CC17 AREfm has consequences for antimicrobial treatment of invasive 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 invasive ampR enterococci. 8 laboratories did not have the historical information

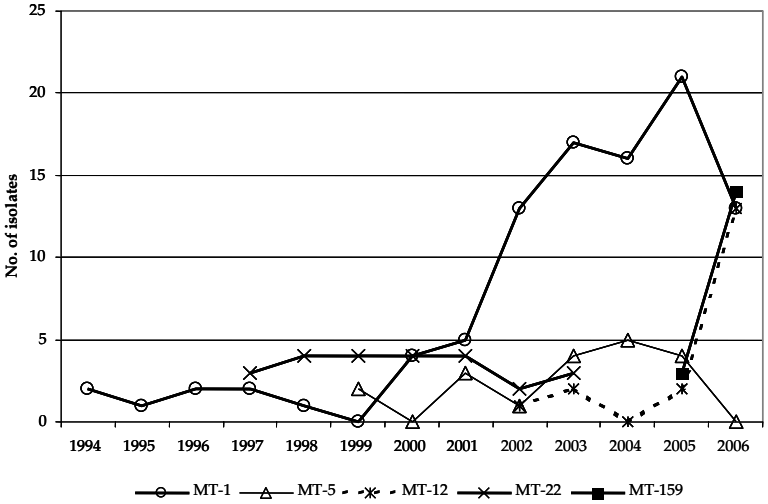

Figure 5. Annual distribution of five predominant MLVA types (MT).

computerized and 30 hospitals never responded to our (once repeated) request. As we failed to have information from all laboratories, some selection bias cannot be fully excluded. Yet, only one of the participating hospitals (large non-university) had identified a nosocomial outbreak with AREfm before our study request. On the other hand, three hospitals that did not participate reported emergence of AREfm infections (all MT-159) in 2006 (personal communication).

Furthermore, two of the participating laboratories could not provide information on isolation sites and, therefore, some isolates might not reflect invasive infections. However, this would account only for urine isolates, as surveillance for asymptomatic carriage with ampR enterococci had not been performed in any hospital. Few laboratories had stored invasive 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 reported proportions of AREfm probably reflects an unbiased estimate. For all these reasons we consider the current study as a reliable reflection of the enterococcal epidemiology in the Netherlands.The increase and replacement of AREfm was most pronounced in university hospitals and large non-university hospitals (>500 beds), which probably reflects differences in patient population, as compared to smaller hospitals. Haematology and transplant patients are generally considered at highest risk for enterococcal bacteraemia (5,29). In our hospital, the increase of AREfm bloodstream infections was associated with increased fecal carriage of AREfm among hospitalized patients (29). Point-prevalence studies revealed intestinal colonization with AREfm in up to 35% of hospitalized patients, especially among high-risk patients on haematology and nephrology wards. Although colonization data are absent

for other centers, endemicity of intestinal colonization with AREfm has probably been established in multiple hospitals in the Netherlands.

MLVA typing revealed that four genetically highly related types caused the nationwide AREfm emergence. MT-159 *E. faecium* isolates first appeared in one hospital in 2005, with documented presence in 5 hospitals in 2006. However, outbreaks of AREfm documented in three other hospitals and not included in this study, were also caused by MT-159 isolates (data not shown). MLST typing 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 (1,13,15,22).

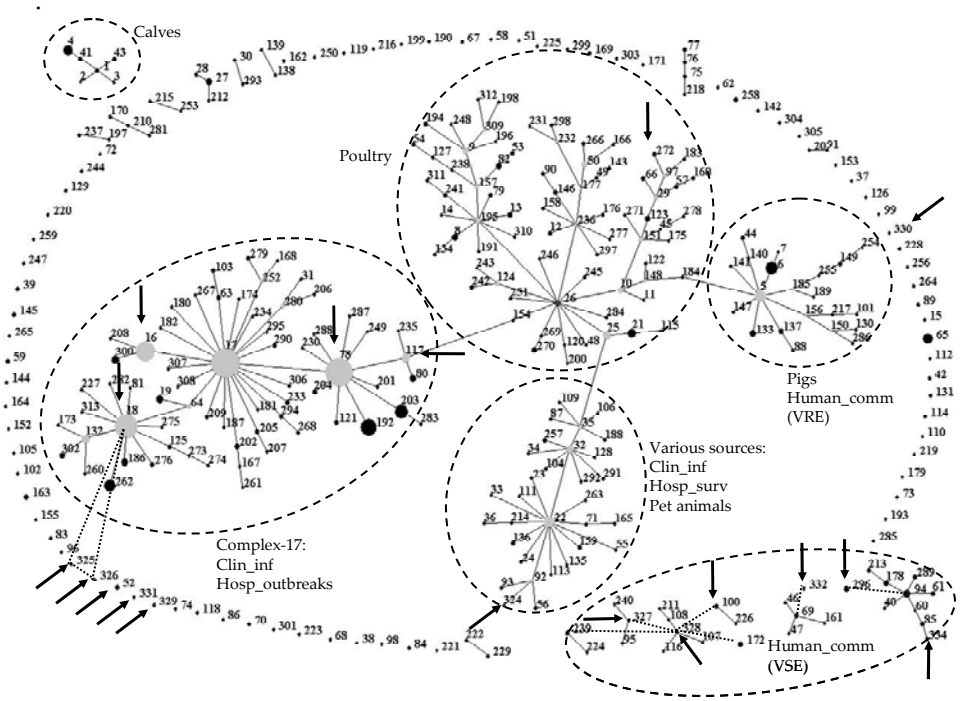

Figure 6. eBURST clustering of 18 MLST profiles, indicated with an arrow, representing 27 isolates from current study, with 313 MLST profiles representing 855 *E. faecium* isolates from the database (www.mlst.net). Each ST is represented as a node, the relative size of each node is indicative for their 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. Source specific clusters of STs are indicated, including CC17 comprising hospital outbreaks and clinical isolates. Annotations: Clin_inf: isolates from clinical sites (mainly blood) from hospitalized patients; Hosp_outbreak, hospital outbreak isolates; Hosp_surv: faeces isolates from hospitalized patients without an enterococcal infection and not associated with an enterococcal hospital outbreak; Human_comm: faeces isolates from human volunteers not connected to hospitals.

As CC17 is based on MLST typing of *E. faecium* isolates, we previously proposed criteria to identify CC17 specific MLVA profiles (28). Comparison of the different repeat-combinations for VNTR-7, -8 and -10 with the obtained 61 MLVA profiles revealed that 86% of the isolates belonged to CC17. MLST typing on representative isolates of new MTs will probably result in extension of CC17 specific MLVA profiles.

Interestingly, the majority (57%) of *esp* positive isolates were found from 2004 on, and this gene was contained in MT-1, and its genetically related variants MT-12 and -159. We consider the *esp* gene as a marker of a putative pathogenicity island (16). 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 (21), which might contribute to increased ability to spread and causing 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 (30). Therefore a role of Esp in the early stage of colonization and subsequent infection has been hypothesized (30).

MLST typing 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 (28,31). The observation that particular MLVA-types, like the MT-12 isolates from this study, is represented by different MLST types and vice versa has been reported before (28), and probably results from differences in the frequency in occurrence of changes in repeat numbers as compared to DNA polymorphisms, mutation and recombination, in housekeeping genes.

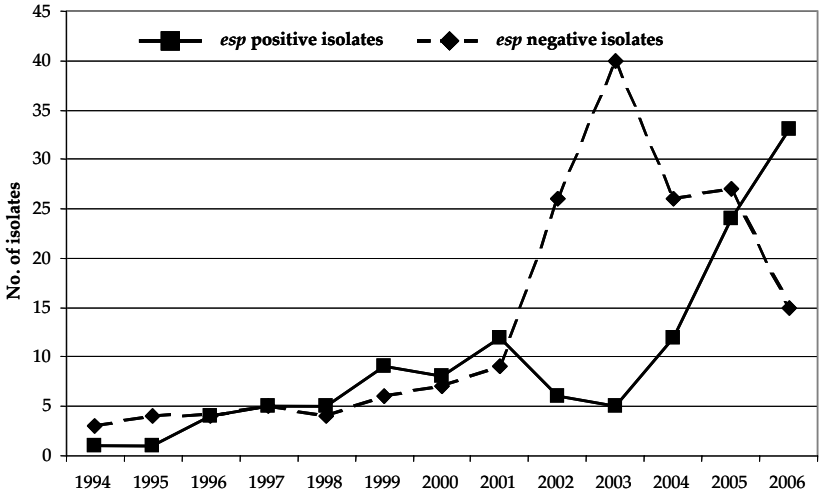

Figure 7. Comparison of the annual distribution of *esp* positive and negative isolates.

MLVA typing of 40 ampS *E. faecium* isolates revealed incomplete MLVA profiles. Southern blot hybridization of three representative isolates confirmed absence of at least one of the VNTR regions (data not shown). MLST typing of 10 MLVA non-typable isolates confirmed that the ampS *E. faecium* isolates are not linked to CC17, but clustered with other MLVA non-typable ampS *E. faecium* isolates, which were not involved in hospital outbreaks.

To our knowledge this is the first nationwide study in Europe on the molecular epidemiology of AREfm. The emergence of CC17 AREfm, resulting in changing *E. faecalis*/*E. faecium* ratios among bloodstream isolates and with 78% of *E. faecium* isolated being ampicillin resistant 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 horizontal transfer of vancomycin resistance genes (12,27,29), mutations leading to resistance for linezolid (14,24,32) or so far not described resistance to daptomycin (20,23).

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