Molecular Epidemiology of *Enterococcus faecium*

from commensal to hospital adapted pathogen

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Chapter 1

General introduction

General

Enterococci are widespread in nature and commonly found in alimentary tracts of humans and other animals as well as in soil, water and food. In human adults, enterococci account for 1% of the intestinal microflora (90). Enterococci are facultative anaerobic, catalase-negative gram-positive cocci that occur singly, in pairs or as short chains. The optimum growth temperature is at 35°C, but the growth temperature can range from 10 to 45°C. All enterococci grow in broth containing 6.5% NaCl and hydrolyze esculin in the presence of 40% bile salts (bile-esculin medium) (32). Among the enterococcal species, *Enterococcus faecalis* and *Enterococcus faecium* are the most commonly encountered species in human faeces (67,74). Although enterococci were for years considered as harmless inhabitants of the gut flora, they are now among the leading causes of nosocomial infections of humans. Originally, the majority of clinical infections like bacteraemia, endocarditis, urinary tract and surgical wound infections were caused by *E. faecalis* (80-90%), while *E. faecium* was found much less frequently (isolated in almost 10% of the infections) (40,45,58,76,85). However, the ratio *E. faecalis* to *E. faecium* infections changed in favor of *E. faecium* in the US in late 1990s (66,75,103). Other enterococcal species which occasionally cause infections in humans are *Enterococcus durans*, *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus hirae*, *Enterococcus gallinarum*, *Enterococcus raffinosus* and *Enterococcus muntdii* (40,67,85).

Antimicrobial resistance in enterococci

Antimicrobial resistance in enterococci can be divided in two classes, intrinsic resistance and acquired resistance (Table 1). Intrinsic resistance is due to either lack of target sites for the antibiotic drug or insufficient penetration of the drug to the intracellular target site. For example, enterococci don't posses penicillin binding proteins (PBPs), which bind cephalosporins with high affinity (37,73). Furthermore, as a result of poor permeability of the enterococcal cell wall, aminoglycosides are unable to reach their target site (64). More important in the emergence of resistance is the ability of enterococci to acquire resistance through either chromosomal mutations or genetic exchange of mobile elements like transposons or plasmids (123). For example, mutations in the DNA gyrase or topisomerase genes reduce the affinity of quinolones for these genes (96). In *E. faecalis* and *E. faecium* many different transposons and plasmids have been identified conferring resistance to a wide variety of antimicrobial drugs, including vancomycin, streptomycin, kanamycin, tetracycline, gentamycin and erythromycin (113). These resistance genes are present in combinations on large

composite elements or as single genes. It has been hypothesized that *E. faecium* plays a central role in the acquisition, conservation and transfer of antimicrobial resistance genes among bacteria (116).

Ampicillin resistance

Enterococci are intrinsic resistant to ß-lactam antibiotics due to low affinity of their penicillin binding proteins (PBP) to ß-lactam agents (Table 1) (34,48,122). They possess at least five and sometimes more than nine different PBPs (121). The level of intrinsic resistance differs among the ß-lactam antibiotics. Generally, penicillins (e.g. ampicillin) have the highest activity, carbapenems slightly lower and cephalosporins have the lowest activity (48,67). Except for a few ß-lactamase producing *E. faecalis* isolates identified in the US (67,69,70), high level ampicillin resistance is mainly found in *E. faecium* isolates derived from clinical specimens. High level ampicillin resistance in *E. faecium* is due to either alterations by mutations in PBP5 resulting in even lower affinity for ampicillin (5,56,86,126) or by overproduction of PBP5 (33,51,126). In 2000, a novel mechanism of ß-lactam resistance has been described in a laboratory mutant of *E. faecium* not involving PBPs (61). In this strain cross-linking during cell wall elongation occurred by a LD-transpeptidation, which by-passes the usual ß-lactam-susceptible DD-transpeptidation (61,62). So far, no clinical isolates with this type of resistance have been reported.

In the US, the first reports on increase of infections and outbreaks due to ampicillin resistant *E. faecium* (AREfm) were published in the early 1980s (22,41,45,67). In several European countries a similar increase of AREfm has been observed, but with a 10 year delay (27,35,53,102). No data are available whether a similar increase of AREfm infections has occurred in the Netherlands. Such an increase will have clinical implications for the treatment of infections and will lead to increased use of vancomycin with the threat of increased selection of vancomycin resistant *E. faecium* (VREF).

Glycopeptide resistance

The first clinical isolates of vancomycin-resistant enterococci (VRE, both *E. faecium* and *E. faecalis*) were detected in Europe in 1986 (54,104). Since then, VRE have rapidly spread all over the world. Especially in the US, VRE prevalence rates increased from 0% in 1989 to 28.5% in 2003 (1,3). Consequently, in the early 1990s VRE were already the second most common nosocomial pathogen in

Table 1. Antimicrobial susceptibility of enterococci

	Antibiotic	Species	Mechanism of resistance
Intrinsic resistance	ß-lactams: - penicillins (low level) - carbepenems (moderate level) - cephalosporins (high level)	all enterococci	Low afinity penicillin binding proteins (PBP)
	Aminoglycosides (low level)	all enterococci	Inefficient uptake
	Aminoglycosides (moderate level)	*E. faecium*	Production of chromosomal AAC(6')Ii enzyme
	Trimethoprim-sulfamethoxazole	all enterococci	Inefficacy in vivo due to assimilation of exogenous folates
	Lincosamides and streptogramins A	*E. faecalis, E. avium, E. gallinarum, E. casseliflavus*	Putative efflux
	Glycopeptides (low level)	*E. gallinarum, E. casseliflavus*	Production of D-Ala-D-Ser ending peptidoglycan precursors
Acquired resistance	Ampicillin (high level)	*E. faecium, E. hirae*	Overproduction or alterations of PBP5
		E. faecalis	ß-lactamase (rare)
	Aminoglycosides (high level)	*E. faecalis, E. faecium, E. gallinarum, E. casseliflavus*	Aminoglycosides modifying enzymes e.g. AAC(6')-APH (2")
	Macrolides	most enterococci	Ribosomal methylation
	Chloramphenicol	*E. faecium, E. faecalis*	CAT encoding enzymes
	Tetracycline	*E. faecium, E. faecalis*	Modification of ribosome protein
	Quinolones	*E. faecium, E. faecalis*	Alterations in DNA gyrase and Topoisomerase IV
	Glycopeptides (high level)	*E. faecium, E. faecalis*	Precursor modification

the US (36) and became endemic in many hospitals (68). In Europe, VRE prevalence rates in hospitals are rising since the year 2000 (2,88).

In 1988, French researchers discovered that glycopeptide resistance was plasmid-mediated (54). A few years later, the same group identified that vancomycin resistance was located on a small mobile genetic element labeled transposon Tn*1546*, encoding the VanA phenotype (8). Furthermore, the same year a second phenotype, VanB, was identified on a different mobile element, labeled transposon Tn*1547* (79). Due to these self-transferable transposons and plasmids, dissemination of vancomycin resistance is not only the result of clonal expansion of resistant strains, but also of horizontal gene transfer between strains and even species. Already at that time the potential transfer of these easily movable resistance genes to more pathogenic gram-positive bacteria like methicillin resistant *Staphylococcus aureus* (MRSA) was feared. At that time vancomycin was the last antibiotic to treat patients with MRSA infections. The first high-level vancomycin resistant *S. aureus* (VRSA) was identified in Michigan (US) in 2002 (17). Up till now, five additional VRSA isolates have been identified (6). In one case a vancomycin-resistant *E. faecalis* was a likely source of the *vanA* gene cluster (114), while VRSA may have acquired the *vanA* gene cluster from an *E. faecium* isolate in another case (115).

Vancomycin, as well as teicoplanin, belong to the group of glycopeptide antibiotics. These antibiotics bind with high affinity to the D-alanyl-D-alanine (D-Ala-D-Ala) C-terminus of peptidoglycan pentapeptide precursors and block the addition of pentapeptide precursors by transglycosylation to the nascent peptidoglycan chain, thereby preventing subsequent cross-linking catalyzed by transpeptidation (11,80).

Nowadays, six types of vancomycin resistance have been described in enterococci (Table 2, adapted from Courvalin (23)). Of the six phenotypes, the

VanA and VanB type of glycopeptide resistance are most frequently reported (19,82,87). Sequencing and functional analysis of the genes encoded by *vanA* and *vanB* gene clusters revealed that glycopeptide resistance is due to enzymes that encodes for (i) synthesis of low-affinity precursors, in which the C-terminal D-Ala residue is replaced by D-lactate (D-Lac) or D-serine (D-Ser), thus modifying the vancomycin-binding target and (ii) for elimination of the high-affinity precursors that are normally produced by the host, thus removing the vancomycin-binding target (8,9,15,81).

Clinical epidemiology of vancomycin-resistant enterococci

Although the first clinical VRE were detected in Europe, a remarkable difference exists in the epidemiology of VRE between Europe and the US. In the US colonization of hospitalized patients with VRE rapidly increased in the 1990s, up to the current endemic levels in many hospitals. In parallel, nosocomial VRE infection rates increased as well, while colonization in healthy people appeared to be absent. In Europe, prevalence rates in hospitals have remained much lower and only started to rise since the year 2000 (2,88). It has been suggested that the rapid increase of VRE in the US was due to 5-10 fold higher use of vancomycin in the US compared to five European countries, including France, Italy, Germany, United Kingdom and the Netherlands, which have, in total, a similar number of inhabitants (13,49).

In the Netherlands, VREF outbreaks have been reported in three different hospitals. In all cases, intervention measurements were successful in controlling the outbreak (63,100,112). In contrast to the US where VRE is restricted to hospitals, a large community reservoir of VRE among healthy people and farm animals exists in Europe, which is most probably linked to massive use of avoparcin in animal husbandry (95,105,107-111). Avoparcin is a glycopeptide antibiotic, like vancomycin, and has been used as growth promoter in the agricultural industry since the 1970s in most European countries. Since the presence of a large community reservoir of VRE was thought to pose a threat for VRE transmission into hospitals either by enterococcal strains harboring the vancomycin resistance genes or by horizontal transfer of Tn*1546* from animal strains to human strains, the European Union banned the use of avoparcin in April 1997. Since then, prevalence rates of VRE colonization among farm animals and human volunteers have decreased (4,50,106).

Table 2. Level and type of resistance to vancomycin in enterococci

Resistance	Acquired					Intrinsic
	High level	Variable level	Moderate level	Low level		Low level
Type	VanA	VanB1/B2/B3	VanD	VanE	VanG	VanC1/C2/C3
MIC (mg/L)						
Vancomycin	64-1000	4-1000	64-128	8-32	16	2-32
Teicoplanin	16-512	0.5-1	4-64	0.5	0.5	0.5-1
Conjugation	Positive	Positive	Negative	Negative	Positive	Negative
Mobile element	Tn*1546*	Tn*1547* Tn*1549* -Tn*5382*	Unknown	Unknown	Unknown	Not applicable
Species	*E. faecium* *E. faecalis* *E. gallinarum* *E. casseliflavus* *E. avium* *E. durans* *E. mundtii* *E. raffinosus* *S. aureus*	*E. faecium* *E. faecalis* *S. bovis*	*E. faecium* *E. faecalis*	*E. faecalis*	*E. faecalis*	*E. gallinarum* *E. casseliflavus* *E. flavescens*
Expression	Inducible	Inducible	Constitutive	Inducible	Inducible	Constitutive Inducible
Location	Plasmid Chromosome	Plasmid Chromosome	Chromosome	Chromosome	Chromosome	Chromosome
Modified target	D-Ala-D-Lac	D-Ala-D-Lac	D-Ala-D-Lac	D-Ala-D-Ser	D-Ala-D-Ser	D-Ala-D-Ser

Molecular epidemiology of *E. faecium*

In the late 1990s spread of multi resistant enterococci mainly considered *E. faecium* (19,66,75). Molecular typing methods are essential to determine, in detail, the epidemiology of *E. faecium* and its resistance traits, and to identify outbreaks in hospitals. Furthermore, the recognized presence of *E. faecium* in different ecological niches created an additional need to determine its population structure and genetic evolution.

The first molecular typing methods for enterococci were based on the analysis of plasmid profiles, including plasmid composition and restriction endonuclease analysis of specific plasmids (59,125). In the late 1980s, a new typing method was developed based on analysis of chromosomal DNA restriction endonuclease profiles by pulsed field gel electrophoresis (PFGE) (18), which was soon adapted for enterococci (39,71). Until recently, many laboratories considered PFGE as the "Gold Standard" typing method. However, this method is only suitable to trace transmission of strains in hospital outbreaks. Interlaboratory data exchange is problematic as there is a lack of standardized conditions for electrophoresis and criteria for interpreting PFGE banding patterns (31).

To study the genetic relatedness between epidemiologically nonrelated VREF, amplified-fragment length polymorphism analysis (AFLP) was developed, which allows analysis of polymorphisms among small restriction fragments (119). With this technique particular *E. faecium* genogroups appeared associated with particular hosts, like pigs, calves, poultry and humans. Most importantly though, there were genetic differences between VREF isolated from feces of nonhospitalized persons without infection (genogroup A) and hospital isolates from fecal origin or from infected body sites like blood (genogroup C). Other studies confirmed the existence of these genogroups among vancomycin susceptible *E. faecium* (VSEF) isolates originating from different sources (14,16,21,46,47). Furthermore, AFLP exhibited a discriminatory power comparable to PFGE and discriminated outbreak related isolates from other isolates (46).

Although AFLP appeared to be a robust and fast typing method generating reproducible data within a given laboratory, this method was less suitable for data exchange between different laboratories and for studying the global epidemiology and the evolution of *E. faecium*. For this, a typing method is required, which generates unambiguous data suitable for the development of web-based databases. In 1998, multi locus sequence typing (MLST) was proposed for *Neisseria meningitides* with the aforementioned properties (60). MLST is based on identifying alleles from DNA sequences of internal fragments of housekeeping genes resulting in a numeric allelic profile. Each profile is assigned a sequence type (ST). In addition, an Internet site with the possibility for data exchange was

developed (www.mlst.net), which currently, together with www.pubMLST.org, contains MLST schemes of 38 different bacterial species, including *E. faecium* (43) and *E. faecalis* (84). MLST of 123 isolates, including VREF and VSEF originating from human (nonhospitalized, clinical and hospital outbreak) and animal sources from various countries, confirmed the genogroups as determined by AFLP, including the hospital related genogroup C (43). MLST typing of the hospital related isolates revealed that the outbreak isolates clustered in a sub-population designated lineage C1, which was subsequently confirmed in many studies performed world wide (12,21,25,52,55,94,117).

A more detailed study on the population structure and evolution of *E. faecium* is needed for better understanding of the worldwide epidemiology of *E. faecium*. Furthermore, the recognition of hospital adapted *E. faecium* subpopulation created a need for rapid identification and typing of *E. faecium*, in order to better target infection control measures in hospitals.

Molecular characterization of DNA polymorphisms in the *vanA* gene cluster of Tn*1546* in isolates from humans and animals revealed high degrees of DNA polymorphisms due to point mutations, deletions and insertions of different insertion sequences e.g. IS*1216V* and IS*1251* (26,44,89,110,120,124). Identical Tn*1546* variants among VREF recovered from farm animals and humans were identified, which could be a result of either colonization of animal-derived VREF in humans or transfer of Tn*1546* from animal VREF to human enterococcal isolates.

Virulence determinants in *E. faecium*

In contrast to *E. faecalis*, little is known about virulence of *E. faecium* (38). Many clinical isolates of *E. faecium* are resistant to phagocytosis by neutrophils (7), which might be considered a pathogenic property.

Other putative virulence factors are the secreted antigen SagA (99) and a surface exposed antigen designated Acm (72). Both antigens are able to bind to human extracellular matrix proteins. In contrast to the specific collagen-binding adhesin Acm, SagA has broad-spectrum binding to fibrinogen, collagen type I, collagen type IV, fibronectin and laminin. Although the exact role of both antigens in the pathogenesis of *E. faecium* infections is not well understood, adherence to extracellular matrix proteins might be the first step in colonization of the host.

In *Caenorhabditis elegans*, *E. faecium* produces hydrogen peroxide at levels that cause cellular damage (65). Additional studies are necessary to investigate the relevance of hydrogen peroxide production by *E. faecium* in the human host.

Gelatinase is an extracellular zinc mettaloprotease, which contributes to *E. faecalis* virulence in some animal models and is regulated through a cell-density-dependent manner by the *fsr* operon (77,78). Recently, dissemination of gelatinase was also described in *E. faecium* (57).

The identification of a hospital adapted *E. faecium* subpopulation raised the question whether this population contained specific traits, which contribute to increased abilities in spread and/or infections among hospitalized patients. Screening of human and animal isolates for the presence of the *esp* gene, which has been associated with increased virulence and biofilm formation in *E. faecalis* (92,93,97,98,101), revealed that in *E. faecium* the *esp* gene is restricted to hospital-derived isolates belonging to the hospital subpopulation (10,13,20,29,30,42,118). Interestingly, in *E. faecalis* this gene is contained on a pathogenicity island (91) and was identified among clinical and animal derived isolates (24,28,42,93). Analysis of the up- and downstream regions of the *E. faecium esp* gene are necessary to determine whether, as in *E. faecalis*, the *esp* gene in *E. faecium* is contained on a pathogenicity island.

In 2003, another putative virulence gene, hyaluronidase (hyl_{Efm}) with homology to the same gene in *Streptococcus pyogenes* and *Streptococcus pneumoniae* was described to be enriched among clinical *E. faecium* isolates (83). Although the presumed function of hyaluronidase in *E. faecium* is still unknown, in *S. pneumoniae* it is suggested that hyaluronidase may contribute to the invasion of the nasopharynx.

Conclusion and aims of the thesis

The recognition of a hospital adapted *E. faecium* subpopulation, which had apparently spread worldwide, and which was, amongst others, characterized by the presence of the *esp* gene, lead to the following research questions:

(i) Can we develop (and validate) rapid identification and typing schemes for *E. faecium*?

For this the accuracy to identify enterococci of current phenotypic tests, automated microbiology systems, API system and a newly developed identification method designated Raman spectroscopy were evaluated (chapter 2). In addition a rapid, robust and cheap typing method (MLVA) allowing the study of genetic relatedness and epidemiology of *E. faecium* with the possibility of interlaboratory data exchange via Internet was developed (chapter 3) and compared to the currently considered "Gold standard" for enterococcal genotyping Pulsed Field Gel electrophoresis (chapter 4).

(ii) Is the *esp* gene in *E. faecium* contained on a pathogenicity island as it is in *E. faecalis* (chapter 5)?

(iii) What is the population structure of *E. faecium* and can we determine evolutionary steps that have lead to the hospital adapted subpopulation (chapter 6)?

(iv) What is the epidemiology of ampicillin resistant *E. faecium* in our hospital (UMCU) (chapter 7) and in the Netherlands (chapter 8)?

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Evaluation of Rapid Phenotypic tests, BD Phoenix, VITEK 2, APIweb and Raman spectroscopy for the Identification of Enterococci

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Abstract

A panel of five rapid phenotypic tests (RPT), the Phoenix, the VITEK 2, the API Strep 20 in combination with the new APIweb and a relatively new phenotypic test, Raman spectroscopy, were evaluated for the identification to species level of 95 non-repeat enterococcal isolates, comprising 7 different species. The RPT panel, a newly designed combination of 5 old tests (reduction of litmus milk, acidification of arabinose, hydrolysis of L-arginine, pigment production, and motility) is a cheap and simple method that provides identification within 4 hours. As reference method, a genotypic test based on the sequence of the *rpoA* gene was used. The accuracy to correctly identify enterococci to species level varied from 82% for APIweb, 87% for both automated microbiology systems to 92% for the RPT, which performed significantly better than APIweb (p=0.05). The best method for the identification of *E. faecium* and for discrimination between high-level resistant (*vanA*/*B*) *Enterococcus faecium*/*Enterococcus faecalis* and intrinsic resistant (*vanC*) *Enterococcus casseliflavus*/*Enterococcus gallinarum* was the RPT with a sensitivity and specificity of 94%/100% and 99%/100%, respectively. For the Phoenix these percentages were 85%/96% and 97%/100% respectively, and for the VITEK 2 85%/98% and 99%/92% respectively. The APIweb revealed despite high sensitivities (96% and 99%, respectively), the lowest specificities among the different methods (85% and 42%, respectively). Although not yet commercially available, Raman spectroscopy revealed a promising rapid phenotypic test with high sensitivities (98% and 99%, respectively) and somewhat lower specificities of 94% and 83%, which will probably increase with expansion of the reference database. To conclude, RPT revealed a highly reliable, very fast and cheap method. As a consequence VRE infection control measures (implementation or ending) can be executed one day earlier, resulting in improvement of efficiency and lowering of costs. Furthermore adequate differentiation of (*vanC*) *E. casseliflavus*/*E. gallinarum* from other species is clinically important since vancomycin treatment failure has been associated with the presence of a *vanC* gene.

Introduction

The identification of hospitalized patients infected or colonized with vancomycin-resistant enterococci (VRE) has become an important component of infection-control programs aimed at minimizing cross-transmission of these organisms (1). Nowadays, six phenotypes of glycopeptide resistance have been reported (9), from which the transferable VanA and VanB phenotypes are predominantly found among the clinically relevant *Enterococcus faecalis* and

Enterococcus faecium isolates. VanA phenotype strains are characterized by high-level resistance to both vancomycin and teicoplanin, whereas VanB phenotype strains are resistant to variable levels of vancomycin only. However, the occasionally disease causing *Enterococcus casseliflavus* and *Enterococcus gallinarum* express intrinsically low-level resistance to vancomycin due to a chromosomally located *vanC* gene (9). The differentiation between *E. faecalis*/*E. faecium*, that can serve as host for *vanA*/*B* genes and *vanC*-VRE is relevant since in contrast to *E. faecalis*/*E. faecium*, *vanC*-VRE have not been implicated in outbreaks and appear to be of minimal concern from an infection-control point of view (31). Also from the clinical perspective it is relevant to differentiate *E. casseliflavus* and *E. gallinarum* from other species. Vancomycin treatment failure has been associated with *vanC* gene encoded low-level vancomycin resistance in humans as well as in animal models of experimental endocarditis (14,18,21). Furthermore, several reports have been published reporting failure or breakthrough bacteraemia during vancomycin therapy with *in vitro* susceptible strains (28,29). Consequently, vancomycin therapy for infections caused by *vanC* VRE is generally regarded inadequate (8). Susceptibility testing alone is insufficient to detect VanC-VRE because *vanC* encoded low-level resistance may not be detected using the CLSI breakpoints.

Another reason for adequate species identification is the worldwide increase of *E. faecium* as causative agent of clinical enterococcal infections and hospital outbreaks (35). Population biology and genetic evolutionary studies using multilocus sequence typing (MLST) have identified a distinct *E. faecium* genetic subpopulation, designated complex-17, comprised of highly transmittable isolates well adapted to the hospital environment and characterized by high-level resistance against ampicillin and ciprofloxacin (20,35). Since complex-17 isolates may require specific infection control measures an adequate identification of *E. faecium* is warranted.

In the routine clinical microbiology laboratory of the University Medical Center Utrecht (UMCU) identification of enterococci to species level was only applied to invasive isolates and VRE screening isolates by the API 20 Strep using Apilabplus V3.3.3 for interpretation. Review of the results of the API 20 Strep in combination with the susceptibility patterns revealed an unexpected high number of ampicillin resistant *E. casseliflavus*. Further analysis showed that these isolates were misidentified and appeared to be *E. faecium*. This finding prompted the current study for the evaluation of API 20 Strep in combination with APIweb V1.2.1 for interpretation, a panel of six phenotypic tests, two automated microbiology systems, VITEK 2 and Phoenix, respectively, and a relatively new phenotypic method Raman spectroscopy (19,24-26). A genotypic test based on the sequence of the *rpoA* gene was used as reference method (27).

Materials and methods

Bacterial isolates and growth conditions

The bacterial isolates used in this study consisted of 34 isolates from our strain collection, including 7 *E. faecium*, 11 *E. faecalis*, 3 *E. casseliflavus* and 5 *E. gallinarum* identified by a species specific PCR targeted on the *ddl* gene as described previously (11,12) and 3 *E. avium*, 2 *E. hirae*, 1 *E. durans* and 2 *Enterococcus* species based on identification using amplified fragment length polymorphism (AFLP) (34). Furthermore, 61 non-repeat clinical isolates were identified as *E. faecium* (25), *E. faecalis* (13), *E. casseliflavus* (16), *E. gallinarum* (1), *E. avium* (5) and *E. durans* (1) using the API 20 Strep (Apilabplus V3.3.3). Twelve of the 16 *E. casseliflavus* isolates as well as the one *E. gallinarum* isolate had been tested in the routine laboratory as ampicillin resistant. All strains were grown overnight on blood agar plates at 37°C.

Reference species identification method

A molecular identification method based on sequence analysis of the α-subunit of bacterial RNA polymerase (*rpoA*) gene (27) was used as reference method with the following modifications. Instead of a time consuming DNA purification step, crude lysates were prepared from bacterial isolates grown overnight on blood agar plates at 37°C. Approximately seven CFUs were suspended in 20 µl lysis buffer (0.25% SDS, 0.05 N NaOH) and incubated at 95°C for 5 min. The cell lysate was spin by short centrifugation and diluted with 180 µl buffer (10 mM Tris-HCl, pH 8.5). After thoroughly mixing, another centrifugation for 5 min at 16,000 x *g* was performed to remove cell debris. Supernatants were frozen at -20°C until further use.

No PCR product was obtained on crude lysates with the previously described primers for the amplification of the *rpoA* gene applied to purified DNA (27). Therefore, PCR conditions were optimized for crude lysates with newly developed primers based on internal fragments of the in Genbank deposited *rpoA* reference sequences, which included 28 different enterococcal species (27). Due to

Table 1. Overview of *rpoA* primers for the multiplex PCR

Primer	Primersequence
rpoA.1F	G G G A A T T C C C T A C G T C G
rpoAefs.1F	C T T T A G G **T** A A **C** T C **T** C T A C G T C G
rpoAegal.1F	G G **A** A A T T C C **T** T A C G T C G
rpoA.1R	T T C G A C C A T G A T T T C A G C
rpoAefs.1R	T T C **C** A C C A T G A T T T C A G C
rpoAegal.1R	T T C **C** A C C A T G A T **C** T C A G C

DNA polymorphisms in the *rpoA* gene a multiplex approach including 3 different primer sets was developed (Table 1). Five µl of the crude lysate was used in the PCR reaction. Reactions were performed in 25 µl volumes with HotStarTaq Master Mix buffers from Qiagen (Qiagen inc.), including 15 pmol per primer. The PCR program comprised of 15 min at 95°C, 30 cycles of 30 sec at 94°C, 30 sec at 52°C, 1 min at 72°C and finally 7 min at 72°C. Five µl of the PCR mix was checked on agarose gel. Without any further purification, 1 µl PCR product was subsequently sequenced, both strands, using ABI BigDye Terminator Cycle sequencing Ready reaction mix version 3.1 (Applied Biosystems, Foster City, USA) and run on an ABI PRISM 3100 Genetic analyzer (Applied Biosystems) according to manufacturers instructions. Raw data were analyzed using BioNumerics software (version 4.5; Applied Maths, Sint-Martens-Latem, Belgium).

To standardize the *rpoA* sequence analysis, a fragment of 430 bp with fixed start and end points (Table 2) was chosen for both the Genbank reference sequences as well as the test isolates. Sequence alignment and cluster analysis by Unweighted Pair Group Method using Arithmetic averages (UPGMA) of the 430 bp fragments of the Genbank *rpoA* reference sequences resulted in a 100% identical species identification as compared to the entire *rpoA* gene (data not shown). The similarity matrix from the UPGMA clustering was used as reference library for the identification of test isolates.

Table 2. Start and end points for the analysis of the *rpoA* sequences

Startpoint *rpoA* sequence	Endpoint *rpoA* sequence	Species
TCAAAGGTGT	GAAGCAATGAG	*E. faecium, E. dispar, E. mundtii,*
		E. ratti, E. phoeniculicola
	GAAGCATTGAG	*E. avium, E. raffinosus, E. pseudoavium,*
		E. malodoratus, E. hermanniensis
	GAAGCATTAAG	*E. gilvus*
	GATGCTATGAG	*E. columbae*
TAAAAGGTGT	GAAGCATTAAG	*E. canis*
	GATGCAATGAG	*E. sulfureus*
TTAAAGGTGT	GAAGCCCTTAG	*E. haemoperoxidus, E. moraviensis*
	GAAGCAATGAG	*E. durans, E. villorum*
	GATGCTATGAG	*E. columbae*
TTAAGGGTGT	GAAGCAATGAG	*E. casseliflavus*
	GACGCAATGAG	*E. saccharolyticus*
	GATGCAATGAG	*E. italicus, E. saccharominimus*
TTCCAGGTGT	GAAGCACTAAG	*E. faecalis*
TTAAGGGCGT	GAAGCAATGAG	*E. gallinarum*
TTAAAGGGGT	GAAGCAATGAG	*E. hirae*
TTGTAGGTGT	GACGCAATGAG	*E. cecorum*
TCAAAGGCGT	GACGCGTTAAG	*E. solitarius*

API 20 Strep

All isolates were tested using API 20 Strep system (BioMérieux, S.A. Marcy-l'Etoile, France) according to manufacturers instructions. Identification was performed using APIweb V1.2.1. Identification (ID) was regarded excellent when the probability was ≥ 99.9% and the T index ≥ 0.75, very good ≥ 99% and T ≥ 0.50, good ≥ 90% and T ≥ 0.25 and acceptable ≥ 80% and T ≥ 0. The identification was not reliable when probability was below 80%.

Automated microbiology systems

Two different systems were included in the evaluation, the Phoenix (BD Biosciences, USA) and the VITEK 2 (BioMérieux S.A. Marcy-l'Etoile, France).

PMIC/ID-50 Phoenix panels (combined susceptibility and identification card) were inoculated according to manufacturers recommendations. For this study software version V5.03E/V4.11B was used. Biochemical reactions contained on the panel are read at 20-minute intervals by the instrument. As soon as, as a function of time, the biochemical profile of the test strain compared to the profiles from the Phoenix database is above the 90% probability value, identification results of bacteria are given. The Phoenix does not categorize the accuracy of identification above the 90% probability.

The VITEK 2 system was used as specified by the manufacturer. Specific Gram-positive cards (Vitek 2 GP card) were used for identification and results were interpreted using software version VT2-R04.01. Identification was regarded excellent when the probability was between 96% and 99%, very good between 93% and 95%, good between 89% and 92%, acceptable between 85% and 88% and low when the probability was below 85%.

Phenotypic tests

In total six different phenotypic tests were evaluated. (i) Hydrolysis of L-arginine was determined using arginine dihydrolase (ADH) diagnostic tablets (Rosco, Taastrup, Denmark) and (ii) acidification of arabinose (ARA) (Rosco, Taastrup, Denmark) according to manufacturers instructions. (iii) Reduction of lithmus milk (LM) (Becton Dickinson, Le Pont de Claix, France) was performed as described by Hanson *et al.* (16) and interpreted after 4 and 24 hours. (iv) Acidification of methyl-α-D-glucopyranoside (MGP) (ICN, Ohio, USA) was determined as described by Carvalho *et al.* (6). (v) Motility was determined as previously described using *Enterobacter aerogenes* as positive motile control and *Klebsiella pneumoniae* as nonmotile negative control (32,33). (vi) Yellow pigment was observed using a cotton swab (5). The reaction patterns for the 7 different enterococcal species included in this study were interpreted according to literature (Table 3).

Raman spectroscopy

Raman spectroscopy is a non-destructive optical technique that can be used to analyze the (bio-) chemical composition of a wide variety of samples. The usefulness of this technique to study microbiological samples has been shown earlier (26). For the measurements, all isolates were grown on Mueller Hinton agar (Beckton Dickinson, Franklin Lakes, NJ, USA) for ~24 hours at 35°C. A calibrated 1μl loop full of CFU was suspended in 7.5 μl of sterilized distilled water, 4 μl of this suspension was transferred to a quartz slide (Hellma Benelux, Rijswijk, The Netherlands) and allowed to dry.

Raman spectra were collected using a High Performance Raman Module 2500 (River Diagnostics BV, Rotterdam, The Netherlands) coupled to an automated XYZ-stage (River Diagnostics) and operated using RiverICon software, version 1.63. Samples were excited using a 785 nm diode laser (Sacher Lasertechnik, Marburg, Germany), delivering approximately 150 mW on the sample. The spectrometer was calibrated using the manufacturers guidelines, and the spectral region of 400 to 1800 cm^{-1} with a resolution of ~2 cm^{-1} was used for the analysis. Data analysis was performed using MATLAB version 7.1 (The Mathworks, Natick, MA, USA).

For each isolate, 50 spectra were collected from different locations, using 1 second exposure time on each location. An average of the 50 spectra was used in the analysis.

Identification of isolates is based on comparison of the obtained spectra with a reference database of spectra. Since this method is under development and not commercially available, we used internal validation to estimate the method's identification capabilities. Briefly, the similarity between each sample pair is calculated using the squared Pearson correlation coefficient (R^2) and multiplied by 100 to be expressed as percentages. For each individual sample, the predicted species (i.e. identification) was similar to the sample with the highest R^2 value. Cluster analysis on 83 spectra was performed using MATLAB version 7.1 (The Mathworks, Natick, MA, USA) by the Ward's algorithm.

For practical reasons only the most prevalent species were analyzed using Raman spectroscopy; *E. avium*, *E. durans*, and *E. hirae* were omitted from the analysis.

Table 3. Results of phenotypic tests

Phenotypic test		ADH		ARA		LM		Pigment		Motility		MGP	
Incubation time		4 hours		4 hours		4 hours		4 hours		4 hours		24 hours	
Species	No. of isolates	Expected[a]	% Correctly identified	Expected[b]	% Correctly identified	Expected[c]	% Correctly identified	Expected[d]	% Correctly identified	Expected[e]	% Correctly identified	Expected[f]	% Correctly identified
E. faecium	48	+	100	+	94	-	98	-	100	-	100	-	100
E. faecalis	23	+	100	-	96	+	96	-	100	-	100	-	100
E. gallinarum	8	+	100	+	100	+	88	-	88	+	100	+	100
E. casseliflavus	4	+	50	+	75	+	75	+	100	+	100	+	100
E. avium	9	-	100	+	78	-	100	-	100	-	100	+	67
E. durans	1	+	100	-	100	-	100	-	100	-	100	-	100
E. hirae	2	+	100	-	100	-	100	-	100	-	100	-	100

[a] some *E. faecalis*, *E. casseliflavus* and *E. gallinarum* isolates are ADH negative (6)

[b] some *E. casseliflavus* and *E. gallinarum* isolates are ARA negative (6,16)

[c] some *E. casseliflavus* isolates are LM negative (16)

[d] some *E. casseliflavus* don't produce pigment (6)

[e] some *E. casseliflavus* and *E. gallinarum* isolates are nonmotile (6)

[f] some *E. faecium* and *E. faecalis* isolates are MGP positive, while some *E. gallinarum* isolates are MGP negative (6,7,16)

Abbreviations: ADH, arginine dihydrolase; ARA, arabinose; LM, lithmus milk; MGP, methyl-α-D-glucopyranoside

Table 4. Identification results

		No. of strains that were:				
Method	Species	Tested	Correctly identified	Identified with low discrimination[a]	Misidentified	Accuracy (%)
APIweb vs 1.2.1	*E. faecium*	48	46		*E. gallinarum* (1); *E.hirae*/*E. durans* (1)	96
	E. faecalis	23	23			100
	E. casseliflavus	4			*E. faecium* (2); *E. gallinarum* (1); *Aerococcus viridans* (1)	0
	E. gallinarum	8		3	*E. faecium* (5)	0
	E. avium	9	8	1		89
	E. durans	1	1			100
	E. hirae	2		2		0
Total						82

Phoenix	*E. faecium*	48	41		*E. faecalis* (2); *E. casseliflavus /E. gallinarum* (1); *E.hirae* (2); *E. durans* (2)	85
	E. faecalis	23	21		*E. faecium* (1); *E. casseliflavus /E. gallinarum* (1)	91
	E. casseliflavus [b]	4	4			100
	E. gallinarum [b]	8	8			100
	E. avium	9	7		*E. raffinosus* (1); *Streptococcus uberis* (1)	78
	E. durans	1	1			100
	E. hirae	2	1		*E. faecium* (1)	50
Total						87
VITEK 2	*E. faecium*	48	41	2	*E. faecalis* (2); *E. durans* (3)	85
	E. faecalis	23	23			100
	E. casseliflavus	4	3	1		75
	E. gallinarum	8	7		*E. faecium* (1)	88
	E. avium	9	7		*Pediococcus pentosaceus* (1); *Streptococcus alactolyticus* (1)	78
	E. durans	1	1			100
	E. hirae	2	1		*E. faecalis* (1)	50
Total						87
Rapid Phenotypic tests	*E. faecium*	48	45		*E. faecalis* (1); *E.hirae /E. durans* (2)	94
	E. faecalis	23	21		*E. gallinarum* (1); *E.hirae/E. durans* (1)	91
	E. casseliflavus	4	4			100
	E. gallinarum	8	7		*E. casseliflavus* (1)	88
	E. avium	9	7		*E.* species (2)	78
	E. durans	1	1			100
	E. hirae	2	2			100
Total						92[c]
Raman spectroscopy	*E. faecium*	48	47		*E. gallinarum* (1)	98
	E. faecalis	23	22		*E. faecium* (1)	96
	E. casseliflavus	4	3		*E. gallinarum* (1)	75
	E. gallinarum	8	6		*E. faecium* (1); *E. faecalis* (1)	75

[a] Only APIweb and VITEK 2 provide identification (ID) message

[b] BD Phoenix doesn't discriminate between *E. casseliflavus* and *E. gallinarum*

[c] Significant with APIweb (p =0.05)

Results

rpoA sequence identification

Sequencing of the 430 bp *rpoA* gene fragment of 95 enterococcal isolates revealed a clear discrimination between 7 different species (Figure 1). Using the similarity matrix of the reference sequences, 5 sequences differing in only 1 or 2 bp were identified as originating from *E. faecium*, with one predominant sequence accounting for 40/48 (83%) of the *E. faecium* isolates. Three other sequences with either 1 or 2 bp differences were identified as *E. casseliflavus* specific, while 4/9 isolates that could be identified as *E. avium* based on *rpoA* sequences differed only in 1 bp. *E. hirae* and *E. durans*, *E. faecalis* and *E. gallinarum* were represented by only one *rpoA* sequence and could be clearly distinguished from the other enterococcal species.

No discrepancies between identification based on the *ddl* gene and *rpoA* sequencing were observed. From the isolates identified by AFLP, one *E. durans* isolate revealed by *rpoA* sequencing an *E. faecium* and two *E.* species revealed to be *E. durans* and *E. gallinarum* isolates, respectively.

From the 61 clinical isolates identified by API 20 Strep in the routine clinical laboratory, *rpoA* sequencing confirmed the identification for all *E. faecium* (25) and *E. avium* (5) isolates. Only 1/23 *E. faecalis* was misidentified as *E. faecium*. However, 15/16 (94%) of *E. casseliflavus* isolates were misidentified and revealed twelve *E. faecium* (ampicillin resistant), one *E. avium* and two *E. gallinarum* isolates, respectively, also the single *E. gallinarum* was misidentified as *E. faecium*.

For the evaluation of the different methods the *rpoA*-based species identification of isolates was used.

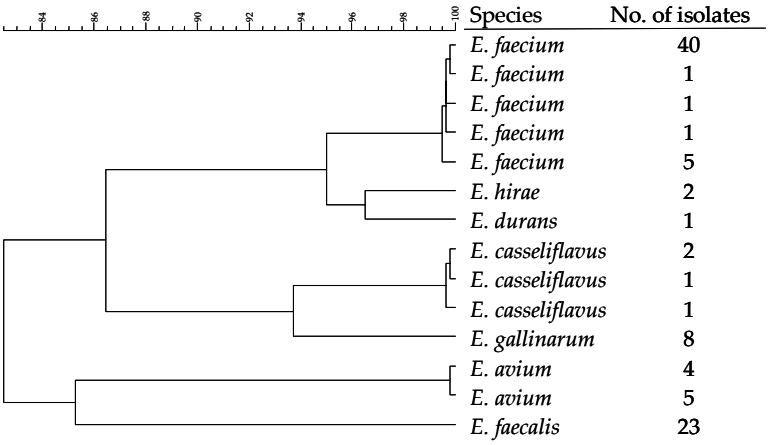

Figure 1. UPGMA clustering of 14 representative rpoA sequences. For each rpoA sequence numbers of isolates are depicted.

API 20 Strep using APIweb

The APIweb performed very well in the identification with an accuracy of *E. faecium* of 96% and *E. faecalis* of 100% (Table 4) The 12 by the Apilabplus V3.3.3 misidentified ampicillin resistant *E. casseliflavus* isolates, were now correctly identified as *E. faecium*. However, the APIweb did not correctly identify all *E. casseliflavus* (4) and *E. gallinarum* (8) isolates. Seven of the *E. casseliflavus* and *E. gallinarum* were identified as *E. faecium*. Despite the high accuracy for the identification of *E. faecium* en *E. faecalis*, APIweb appeared to be the least accurate method for identification of enterococci in general (82%).

Automated microbiology systems

The Phoenix and VITEK 2 appeared to be comparable in their accuracy to identify enterococci, both 87% (Table 4). Both systems misidentified seven *E. faecium* isolates, of which two strain were misidentified by both methods. Incidentally, the two automated systems identified enterococci as other Gram-positive bacteria, mainly streptococci. Furthermore, the Phoenix misidentified 2/23 *E. faecalis* as either *E. faecium* or *E. casseliflavus*/*E. gallinarum* and one *E. hirae* as *E. faecium*. The VITEK 2 misidentified one *E. gallinarum* as *E. faecium* and one *E. hirae* as *E. faecalis*. Finally, both systems were unable to identify 2/9 *E. avium* isolates.

Table 5. Rapid phenotypic test panel

ADH	ARA	LM	Pigment	Motility	Identification
+	+	-	-	-	*E. faecium*
+	-	+	-	-	*E. faecalis*
+	+	+	-	+	*E. gallinarum*
+	+	+	+	+	*E. casseliflavus*
+	-	-	-	-	*E. durans* group[a]
-	+	-	-	-	*E. avium* group[b]
-	-	-	-	-	*E.* species

[a] *E. durans* , *E. dispar* and *E. hirae*

[b] *E. avium* , *E. raffinosis*

Abbreviations: ADH, arginine dihydrolase;

ARA, arabinose; LM, lithmus milk

Phenotypic tests

The phenotypic test results per species are shown in Table 3. All six tests were very easy to perform and interpret. The LM reaction was easy to interpret after 4 hours of incubation, but became unreliable after 24 hours of incubation.

Analysis of the 95 isolates combining ADH, ARA, LM, pigment and motility, further referred to as the rapid phenotypic test panel (RPT) (Table 5) resulted in identification within 4 hours with an overall accuracy of 92% (Table 4), which was better than the automated microbiology systems, but only significant better when compared to APIweb (p =0.05). The MGP test, which needs an overnight incubation step did not enhance the accuracy and was therefore not included in the RPT.

In some cases the individual phenotypic tests revealed a number of a-typical reacting isolates (Table 3). Most pronounced incongruent, though previously described results (6,16) included *E. casseliflavus* isolates that were ADH negative (2), ARA negative (1) or LM negative (1), but combination with the motility and pigment test of the RPT scheme resulted in correct identification of these a-typical reacting *E. casseliflavus* isolates. Other unexpected a-typical reacting isolates were two ARA and three MGP negative reacting *E. avium* isolates, three ARA negative and one LM positive reacting *E. faecium* isolates and one pigment producing *E. gallinarum* isolate (Table 3). Due to these atypical reactions 8 isolates were misidentified, including 3/48 *E. faecium* identified as either *E. faecalis* or *E. hirae*/*E. durans*, 2/23 *E. faecalis* as either *E. gallinarum* or *E. hirae*/*E. durans*, 1/8 *E. gallinarum* identified as *E. casseliflavus* and 2/9 *E. avium* identified as *E.* species (Table 4).

Raman spectroscopy

Clustering based on the averaged spectra of the isolates revealed species-specific clusters, except for 2 isolates (Figure 2), though based on the highest R^2 value 5 isolates were misidentified (Table 4). Based on the highest R^2 value 1/4 (25%) *E. casseliflavus* and 2/8 (25%) *E. gallinarum* were misidentified as *E. gallinarum* and *E. faecium*/*E. faecalis*, respectively. The as *E. faecium* misidentified *E. gallinarum* isolate clustered together with another *E. faecium* isolate and as a consequence, based on the highest R^2 value, this *E. faecium* isolate was misidentified as *E. gallinarum*, although this isolate clustered among the other *E. faecium* isolates. Finally, one *E. faecium* isolate was misidentified as *E. faecalis*, while all *E. faecalis* isolates were correctly identified.

The relatively high accuracy for the identification of *E. faecium* and *E. faecalis* was with 98% and 96%, respectively, comparable to APIweb, but higher when compared to both automated microbiology systems and RPT (Table 4). The relatively low accuracies for *E. casseliflavus* and *E. gallinarum*, both 75%, were probably due to low numbers of isolates tested and hence the limited reference spectra available. The overall accuracy was excluded for comparison with the other identification methods, while *E. avium*, *E. hirae* and *E. durans* were not tested with Raman spectroscopy.

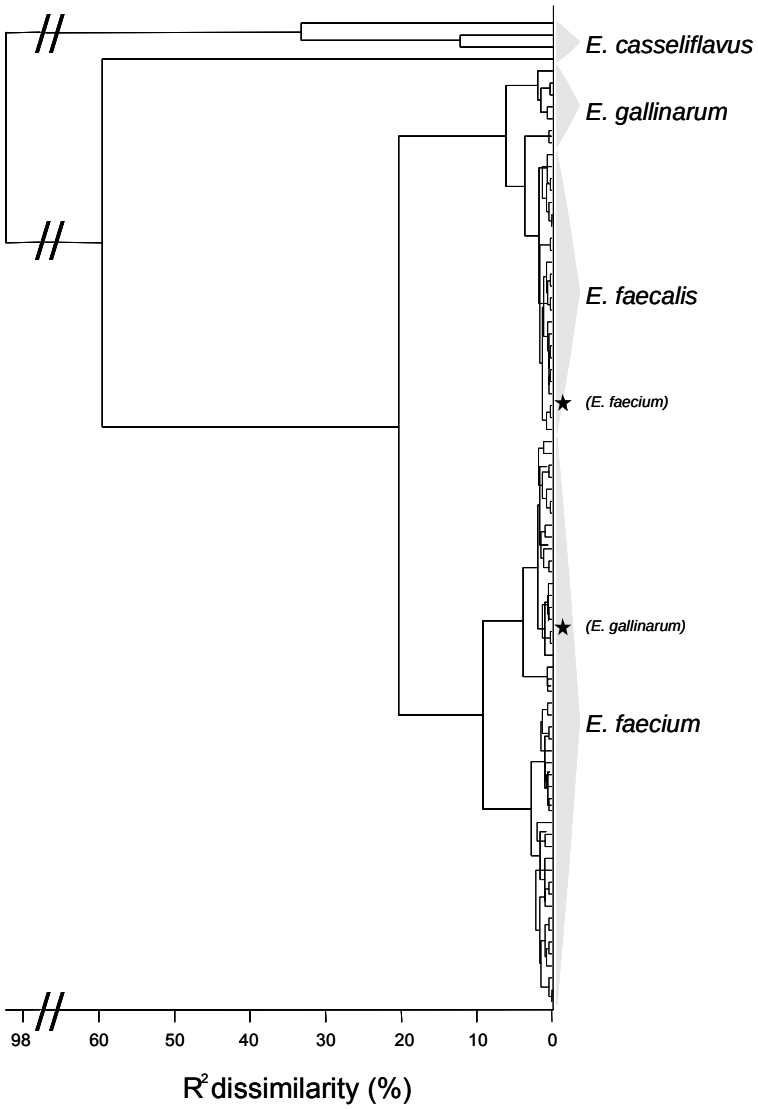

Figure 2. Cluster analysis of 83 Raman spectra using Wards algorithm. Marked isolates were misidentified

Comparative analysis

Comparison of the characteristics of the different identification methods revealed a relatively comparable hands-on time, including preparation of suspensions and interpretation of identification result (Table 6). For the automated microbiology systems and RPT the hands-on time was 4 to 5 minutes, while Raman spectroscopy and API Strep 20 needed 10 to 15 minutes. In contrast, the incubation time varied from 1 minute for Raman spectroscopy to overnight incubation for API Strep 20 and the Phoenix. Technically, for Raman spectroscopy

Table 6. Comparison identification methods

				Identification of *E. faecium*		*E. faecium* /*E. faecalis* vs *E. casseliflavus*/*E. gallinarum*	
Method	Hands-on time	Incubation time	Costs ($)[a]	Sensitivity	Specificity	Sensitivity	Specificity
APIweb	15 Minutes	4-24 Hours	8.10	96	85	99	42
Phoenix	5 Minutes	~24 Hours	6.72	85	96	97	100
VITEK 2	4 Minutes	~ 5 Hours	9.18	85	98	99	92
Rapid phenotypic tests	5 Minutes	4 Hours	1.30	94	100	99	100
Raman spectroscopy	10 Minutes	1 minute	[b]	98	94	99	83

[a] Only materials, purchase of VITEK 2, Phoenix and Raman spectrometer excluded

[b] Unknown, method under development

there is no incubation time, just the measurement time. Only materials costs were included for the comparison and revealed that, compared to RPT both identification cards and API Strep 20 were relatively expensive. Raman spectroscopy was not included, since this method is not yet commercially available.

Identification of *E. faecium*

Sensitivities and specificities of the different methods to identify *E. faecium* revealed that both the RPT (94% and 100%, respectively) and Raman spectroscopy (98% and 94%, respectively) performed very well (Table 6). Both automated microbiology systems revealed the lowest sensitivity with 85% due to high numbers of false-negative *E. faecium* identifications. Although the sensitivity of APIweb was high (96%), the specificity was the lowest (85%) among the methods due to high numbers of false-positive *E. faecium* identifications.

Differentiation between *E. faecalis*/*E. faecium* and *vanC*-VRE

The RPT was superior to the other methods in distinguishing *E. faecalis*/*E. faecium* from *vanC* positive *E. casseliflavus*/*E. gallinarum*, with a sensitivity of 99% and a specificity of 100% (Table 6). The Phoenix also exhibited 100% specificity but a lower sensitivity (97%). Although, Raman spectroscopy revealed a comparable sensitivity (99%), the specificity was relatively low (83%) due to false-positive *E. gallinarum* and *E. casseliflavus* isolates. Despite the high sensitivity of APIweb (99%), the specificity was very low, 42%.

Discussion

Here, we report on the performance of API 20 Strep using APIweb, VITEK 2, Phoenix, Raman spectroscopy and a new developed rapid phenotypic test panel (RPT) to identify enterococci to species level.

For identification the RPT scored better than the automated microbiology systems, but only significant better when compared to APIweb. Furthermore, of

the routine laboratory tests the RPT was the most rapid and reliable method to distinguish *E. faecium*/*E. faecalis* from *E. casseliflavus*/*E. gallinarum* as well as the most simple and cheapest method.

This is the first study, where the routine phenotypic tests as API or automated microbiology systems were compared to a sequence-based reference method with a proven high discriminatory power to differentiate enterococcal species (28). This reference method was a modification of a previously described sequence-based method targeted on the *rpoA* gene using degenerated primers on purified DNA (27). For this study the protocol was modified for crude DNA lysates to reduce time-consuming DNA purification steps using a multiplex PCR approach including species-specific primers. DNA sequencing of 95 isolates and cluster analysis of an internal fragment with fixed start- and endpoints of *rpoA* clearly discriminated the different species in concordance with the previous results (27). Often 16S rRNA sequencing is used for the identification of bacterial species, but this method is less suitable for enterococci due to low discrimination among the so called *E. faecium* group, comprising *E. faecium*, *E. durans*, *E. hirae* and *E. mundtii* (36). In contrast, the *rpoA* sequencing clearly discriminated the different species from the 16S rRNA *E. faecium* group. Alternatively, a multiplex PCR based on the *ddl* and *van* genes (11) has been used to differentiate between enterococcal species. This PCR, however, is limited to the identification of *E. faecium*, *E. faecalis*, *E. casseliflavus* and *E. gallinarum* only. The identification based on *ddl* gene of the 26 isolates included in this study was confirmed by *rpoA* sequencing. In conclusion, the modified *rpoA* sequencing method is the most accurate molecular method to identify enterococci to species level.

Using the API 20 Strep system in combination with the Apilab V3.3.3 a high number of *E. faecium* isolates were misidentified as *E. casseliflavus* (12/16, 75%) confirming the results of a previous study (37). In the current study, the new APIweb version misidentified only 4% of *E. faecium* as *E. casseliflavus*/*E. gallinarum*. However, now 58% of the *E. casseliflavus*/*E. gallinarum* were incorrectly identified as *E. faecium*. So, introduction of the new interpretation scheme has resulted in an improvement of the identification of *E. faecium* but a deterioration of the identification of *E. casseliflavus*/*E. gallinarum* strains, yielding the API 20 Strep/APIweb system less suitable for species identification in the clinical laboratory.

In this study, the accuracy of 87% for both the Phoenix and VITEK 2 to identify enterococci was either comparable (10,15,23) or lower when compared to other studies (2-4,13,30) and confirmed the reported relatively high degree of false negative *E. faecium* identifications by both Phoenix and VITEK 2 (2,3,10,13,15,23). The relative low accuracy found in this study in comparison with previous studies may be explained by the different reference methods used. All of the

aforementioned studies, except one (4), used API as reference identification method often complemented with one or more phenotypic tests despite previous reports on the poor results by the API systems on enterococcal identification. (17,22,37).

The RPT yielded the highest accuracy in identification (92%). Furthermore, sensitivities and specificities of both the identification of *E. faecium*, 94% and 100% respectively, and the distinction between *E. faecium*/*E. faecalis* and *E. casseliflavus*/*E. gallinarum*, 99% and 100% respectively were superior when compared to the Phoenix and VITEK 2. Only API revealed comparable sensitivities, but due to the high degree of false-positive *E. faecium* identifications this would result in unnecessary infection control measures. Therefore, we propose to use the rapid phenotypic test panel for identification of enterococci as an alternative for the more expensive automated microbiology systems and the expensive as well as time-consuming API. A limitation of this scheme is its inability to discriminate between the genetically related *E. hirae* and *E. durans* or between *E. avium* and *E. raffinosis*. However, in the routine setting differentiation between these species is not clinical relevant. Another advantage of the test is the rapid result within 4 hours. As a consequence VRE infection control measures (implementation or ending) can be executed one day earlier, resulting in improvement of efficiency and lowering of costs. The same is the case for the switch from empirical to directed antibiotic therapy.

Raman spectroscopy is a method under development, but was included in this study to compare its abilities in the identification of enterococci in general, the identification of *E. faecium* and the distinction between *E. faecium*/*E. faecalis* and *vanC* positive isolates to other conventional phenotypic tests. Identification of isolates is based on comparison of spectra of the test isolate with a reference database comprising representative spectra of different species (26). In a previous study, only low numbers of enterococcal isolates were included (19) and therefore the reference database had to be build from the isolates included in current study. The clinically less relevant species with low numbers were therefore excluded in the analysis.

Cluster analyses based on the phenotypic spectra revealed species specific clustering. Compared to the other methods a relatively high accuracy was found to identify *E. faecium* and *E. faecalis*, while a much lower accuracy was found for *E. casseliflavus*/*E. gallinarum*, which was probably due to low numbers of isolates in the reference database. As a result, although the sensitivity for the distinction between *E. faecium*/*E. faecalis* and *vanC* positive isolates was almost 99%, the specificity was relatively low with 83%. In contrast, the sensitivity and specificity to identify *E. faecium* was comparable with RPT. In conclusion, Raman spectroscopy is a promising tool to identify enterococci to species level.

Expansion of the reference database including also the less clinical relevant isolates will further improve identification of enterococci. Finally, further studies are needed to evaluate its capacity to identify outbreak related isolates.

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Chapter 3

Multiple-Locus Variable-Number Tandem Repeat Analysis, a Novel Typing Scheme To Study the Genetic Relatedness and Epidemiology of *Enterococcus faecium* Isolates

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Abstract

Multiresistant *Enterococcus faecium* is a major cause of hospital acquired infections and outbreaks. Here, we describe the development of multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA) as a novel typing method to assess the genetic relatedness of *E. faecium* isolates. Six VNTR loci were used to genotype 392 isolates recovered from different animals and human community, hospital survey, and clinical isolates. From 3 to 13 alleles were found per locus, resulting in 127 different MLVA profiles. Clustering of MLVA profiles confirmed the host-specific genogroups found by multilocus sequence typing (MLST) and showed the grouping of clinical and epidemic isolates that belonged to the MLST-C1 cluster in a distinct MLVA-C1 cluster (sensitivity of 97% and specificity of 90%). Furthermore, the discriminatory power of MLVA is comparable to MLST. MLVA profiles appeared to be relatively stable, since isolates from a single outbreak shared the same MLVA profile, which is a prerequisite when MLVA is used to study hospital outbreaks. Our data show that MLVA is a highly reproducible and portable typing method; in contrast to MLST, it is fast, relatively cheap, and easy to perform. Furthermore, it has the abilities of MLST to recognize genetically related and potential epidemic isolates. Submission of MLVA profiles is possible via a Web-based database for international comparison.

Introduction

During the last decade, vancomycin-resistant *Enterococcus faecium* (VREF) has emerged as an important cause of nosocomial infections, especially in immunocompromised patients (20). VREF are often resistant to almost all available antibiotics, seriously hampering treatment of infections. Emergence of ampicillin resistance in *E. faecium* in the United States in the early 1980s preceded the rapid increase of vancomycin resistance (10, 18, 20). Nowadays, VREF is endemic in many hospitals in the United States, and prevalence rates in European hospitals are rising, with VRE rates above 10% in at least six European countries (3, 4, 9, 15, 26, 32; Annual report of the European Antimicrobial Resistance Surveillance System, 2002 [www.earss.rivm.nl]).

Molecular epidemiological studies of both human- and animal-derived *E. faecium* isolates with amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST) revealed the existence of host-specific genogroups (12, 34). Furthermore, a specific genetic lineage (C1), associated with nosocomial outbreaks and infections and clearly distinct from lineages composed of human community- and animal-derived isolates, was identified. This so-called

epidemic genetic lineage, C1, was further characterized by ampicillin resistance and the presence of the *esp* virulence gene (3, 4, 18). The *esp* gene encodes the enterococcal surface protein (Esp), which was first described for *Enterococcus faecalis* and is thought to be an adhesin involved in colonization of the urinary tract (24, 27). The *esp* gene is located on a pathogenicity island in *E. faecalis* as well as in *E. faecium* (17, 23).

Important for infection control is to improve recognition and early detection of the potential epidemic isolates as determined by AFLP and MLST. A low-cost typing scheme that is rapid, reproducible, easy to perform, with the portable character of MLST and the ability to recognize the epidemic MLST-C1 genogroup isolates would therefore be a useful tool for outbreak management. AFLP, although rapid, has a poor rate of reproducibility, and interlaboratory data exchange is not possible. In contrast, MLST is highly reproducible and is appropriate for data exchange via the Internet (www.mlst.net). However, this method is labor intensive and therefore time consuming and rather expensive. Multiple-locus variable-number tandem repeat analysis (MLVA) is based on differences in the variable number of tandem repeats (VNTR) on multiple loci on the chromosome of bacteria, which can rapidly be detected by PCRs with specific primers based on the flanking regions of the tandem repeats. MLVA fulfills the previously mentioned criteria. Since MLVA types (MTs) are discriminated by gain and loss of discrete repeats, MLVA also provides an unambiguous assignment and nomenclature of genotypes, making it a portable technique suitable for data exchange.

In this study, a MLVA typing scheme based on six different tandem repeat loci was developed for *E. faecium*. Here, we show that MLVA typing is as discriminatory as MLST and able to recognize previously identified host-specific genogroups.

Materials and methods

Bacterial isolates

MLVA was performed with 392 isolates, including isolates from clinical sites like blood, urine, and wounds (126 isolates); hospital surveys (68 isolates); 25 different documented hospital outbreaks (111 isolates); community surveys (17 isolates); and samples from various animals, food, and the environment (70 isolates) (Table 1). Hospital and community survey isolates were derived from fecal samples, and none of these were associated with hospital outbreaks. Hospital outbreak isolates were recovered from clinical sites like blood and urine,

Table 1. Isolates used in this study

Source[a]	No. of isolates	MLVA genogroup	Reference or Source
Community surveillance	17	12A, 3B, 2C	30, 31, 33-35
Clinical isolates	126	3A, 17B, 18C, 84C1, 4R	1, 4-6, 14, 18, 22, 32-35
Hospital surveillance	68	17A, 7B, 17C, 24C1, 3R	4, 14, 19, 26, 32-35
Hospital outbreak Australia-1	2	C1	1, 33
Hospital outbreak Australia-2	2	C1	W. Grubb[b]
Hospital outbreak DK-1	1	C1	25
Hospital outbreak DK-2	1	C1	25
Hospital outbreak DK-3	1	C1	25
Hospital outbreak GR-1	2	C1	21
Hospital outbreak GR-2	3	C1	21
Hospital outbreak GR-3	2	C1	21
Hospital outbreak NL-1-1	32	C1	32
Hospital outbreak NL-1-2	7	C1	32
Hospital outbreak NL-2-1	18	C1	19
Hospital outbreak NL-2-2	4	C1	19
Hospital outbreak NL-3-1	4	R	26
Hospital outbreak NO-1	1	C1	15
Hospital outbreak TZA-1	5	C1	B. Blomberg[b]
Hospital outbreak UK-1	4	C1	14
Hospital outbreak US-1	10	C1	5
Hospital outbreak US-2-1	1	C1	2
Hospital outbreak US-2-2	1	C1	2
Hospital outbreak US-2-3	1	C1	2
Hospital outbreak US-2-4	1	C1	2
Hospital outbreak US-2-5	1	C1	2
Hospital outbreak US-2-6	1	C1	2
Hospital outbreak US-2-7	1	C1	2
Hospital outbreak US-3	5	C1	S. Slaughter[b]
Poultry	13	B	14, 31
Pig	20	16A, 3B, 1C	8, 14, 30
Domestic pet	6	3B, 3C	29
Calf	19	B	34
Miscellaneous	12	1A, 2B, 4C, 3C1, 2R	8, 34
Total	392		

[a] Abbreviations: DK, Denmark; GR, Greece; NL, The Netherlands; NO, Norway; TZA, Tanzania; UK, United Kingdom; US, United States.

[b] Personal communication.

as well as from feces. Computer and statistical analyses were performed on all isolates, including one representative isolate from each outbreak (306 isolates).

Tandem repeat search

A search for tandem repeats in the unfinished genome sequence of *E. faecium* published on the Internet site of the DOE Joint Genome Institute (http://www.jgi.doe.gov/JGI_microbial/html/index.html) was performed using the program repeat finder (http://tandem.bu.edu). From the list of tandem repeats, a selection of 10 different loci was made. This selection was based on the following criteria: (i) minimum repeat size of 20 bp, allowing differentiation of the polymorphic VNTR loci by size on agarose gels, (ii) conservation between the tandem repeats (>90%), and (iii) presence in noncoding regions. Initially the 10 VNTR loci were tested on a set of 72 isolates from different origins designated VNTR-1 to VNTR-10. Eventually, six VNTR loci were used and their characteristics are listed in Table 2.

Table 2. VNTR characteristics and specific primers used in MLVA

Locus name	Repeat length (bp)	Range no. repeats	No. of alleles	% Conservation	Primer sequence	PCR program temperature	Estimated size range (bp)	% Agarose gel
VNTR-1	123	0-8	8	95	F: CTGTGATTTGGAGTTAGATGG R: CATTGTCCAGTAGAATTAGATTTG	30 cycli, 52˚C	250-1012	2
VNTR-2	279	1-14	13	96	F: GATGCTTATTTCCACTGCTTGTTG R: GTTTTACCCTCTCTTTTAAGGTCAATG	TD, 70-60˚C	724-4351	1
VNTR-7	121	1-7	7	98	F: CTATCAGTTTCAGCTATTCCATC R: CTGGTACGAATCAAATCAAGTG	TD, 65-55˚C	416-1021	2
VNTR-8	121	1-7	7	96	F: GGGGAGTGGCAAAAAATAGTGTG R: CAGATCATCAACTATCAACCGCTG	TD, 70-60˚C	237-963	2
VNTR-9	121	1-3	3	93	F: CTGCATCTAATAACAAGGACCCATG R: ACATTCCGATTAACGCGAAATAAG	TD, 70-60˚C	205-447	2
VNTR-10	121	0-3	4	96	F: CCTACAGAAAATCCAGACGG R: TTTTTTCCATCCTCT TGAATTG	TD, 65-55˚C	174-474	2

DNA preparation and VNTR PCR

Bacterial isolates were grown overnight on Columbia blood agar plates. Three colonies of bacterial cells were suspended in 20 µl of lysis buffer (0.25% sodium dodecyl sulfate, 0.05 N NaOH) and incubated at 95°C for 5 min. The cell lysate was spin by short centrifugation and diluted with 180 µl of buffer (10 mM Tris-HCl [pH 8.5]). After the lysate was thoroughly mixed, another centrifugation for 5 min at 16,000 x *g* was performed to remove cell debris. Supernatants were frozen at –20°C until further use.

A total of 2.5 µl of lysate was used in the PCR. Chromosomal DNA was extracted from isolates that did not yield a PCR product with the QiaAmp Blood kit (QIAGEN, Inc., Valencia, Calif.) according to the manufacturer's instructions for gram-positive bacteria, with some minor changes in the lyses of the bacteria. From an overnight culture, 1.5 ml was spin for 2 min, suspended in 200 µl of 10 mM Tris-1 mM EDTA (pH 8.0) and 10 µl of a 50-mg/ml solution of egg white lysozyme (Roche), and incubated at 37°C for 15 min. The bacteria were lysed by

the addition of 30 μl of 10% sodium dodecyl sulfate and 20 μl of a 20-mg/ml proteinase K (Merck) solution and subsequently incubated at 65°C for 1 h. Subsequently, the protocol according to the manufacturer's instructions was used. The PCR conditions were not the same for all of the amplification reaction mixtures (Table 2). In all cases, initial denaturation was at 95°C for 15 min, and a final extension step consisted of 5 min at 72°C. For VNTR-1, 35 cycles, each consisting of 30 s at 94°C, 30 s at 52°C, and 30 s at 72°C, were performed. For VNTR-2, VNTR-8, and VNTR-9, a touchdown (TD) PCR was used that included 10 cycles, each consisting of 30 s at 94°C, 30 s at 70°C down to 60°C, and 30 s at 72°C. The annealing temperature during the first cycle was 70°C and decreased 1°C at each cycle during the next nine cycles. During the remaining 25 cycles, an annealing temperature of 60°C was used. For VNTR-7 and VNTR-10, the initial annealing temperature was 65°C and was decreased to 55°C. Reactions were performed in 25-μl volumes with HotStar *Taq* polymerase and HotStar master mix buffers from QIAGEN. PCR fragments were separated on 1 or 2% agarose gels with a 50-bp, 100-bp, or 1-kb ladder as a size marker (Invitrogen) (Table 2).

Computer analysis of MLVA data

An MLVA profile was created from the number of repeats for each of the VNTR loci. For each MLVA profile, an MT was assigned. Clustering of the MLVA profiles was performed with BioNumerics software (version 3.5; Applied Maths) by the unweighted pair group method using arithmetic averages (UPGMA) with the categorical coefficient of similarity and with the eBURST algorithm described by Feil et al. (7), initially developed for MLST but also suitable for MLVA. This algorithm is implemented as a Java applet at http://eburst.mlst.net.

Statistics

To compare the discriminatory ability of MLVA, MLST, and AFLP, Simpson's index of diversity (*D*) and 95% confidence intervals (CI) were calculated for 78 isolates typed by MLST, MLVA, and AFLP according to the formulas described by Grundmann et al. and Hunter et al. (11, 13). To determine whether MLVA was able to identify *E. faecium* genotypes belonging to the epidemic MLST-C1 genogroup, the sensitivity and specificity of MLVA were calculated with a set of 291 isolates. In addition, positive and negative predictive values (PPVs and NPVs) of different MLVA profile combinations were calculated to determine to what extent isolates belonging to the MLST-C1 genogroup were identified.

Isolate characterization using MLVA via the Internet

Comparable to the MLST Internet site (http://www.mlst.net), an Internet site has been developed for the *E. faecium* MLVA scheme (http://www.mlva.umcutrecht.nl). Through this site, submission of MLVA

profiles and assignment of MLVA types are possible. Furthermore, a database with MLVA profiles and strain information can be queried.

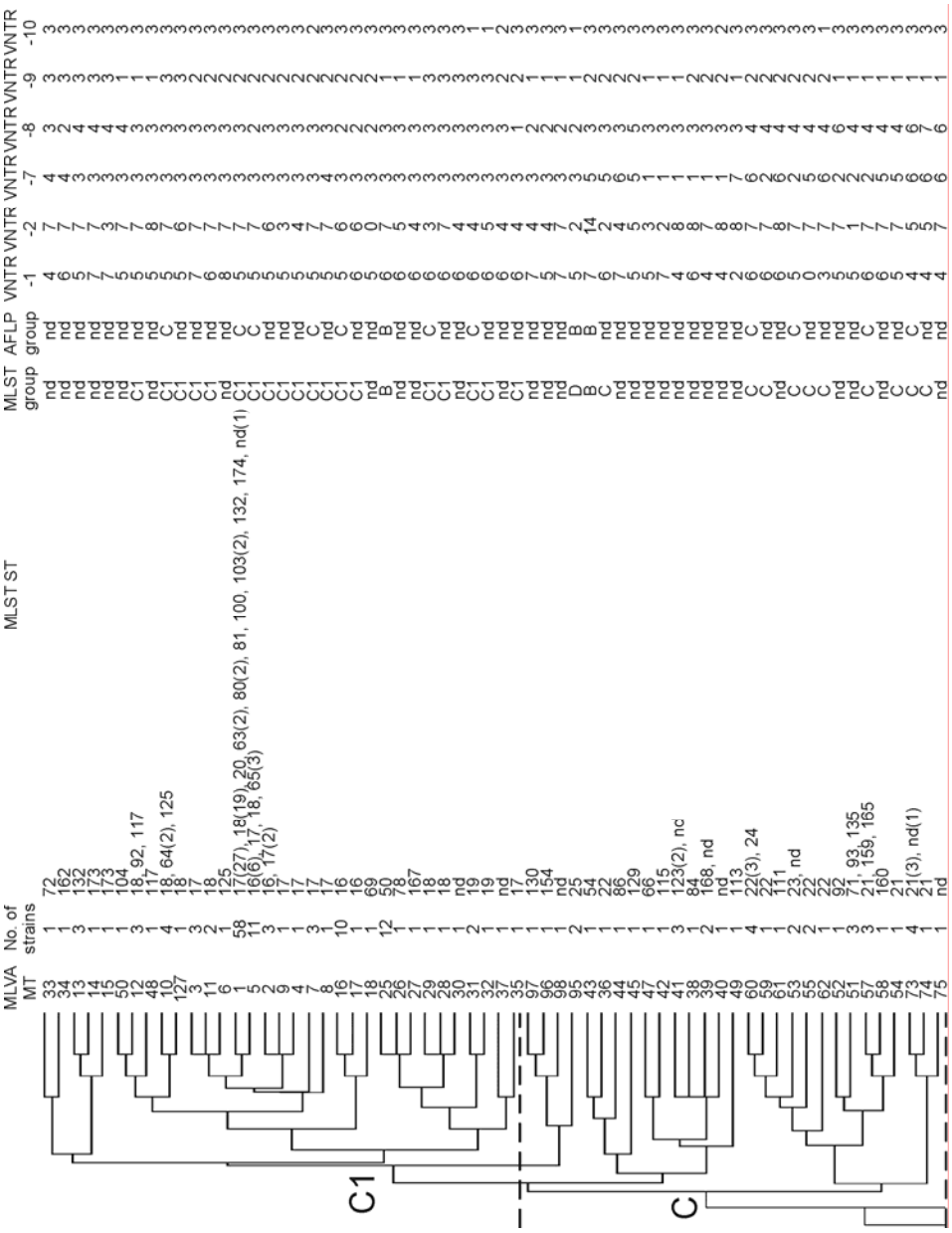
Results

Characteristics of VNTR loci

An MLVA scheme for the molecular typing of *E. faecium* was developed. All 10 VNTR loci were initially tested with a set of 72 isolates of different origins. PCRs were performed on crude bacterial lysate with the exception of the VNTR-2 PCR, which was also performed on QiaAmp-purified DNA. None of the 10 VNTR PCRs yielded PCR products when *E. faecalis* DNA was used as a template. Four of the 10 VNTR loci were unsuitable for typing purposes, due to insufficient polymorphism (one locus) and absence in a number of isolates (three loci). The remaining six VNTR loci were used to type the entire strain set. None of the six VNTR loci were found within open reading frames, and they were present on different contigs of the preliminary genome sequence, indicating that the six VNTR loci are probably scattered over the chromosome. The repeat size of the six VNTR loci ranged from 121 to 279 bp, and the number of alleles ranged from 3 for VNTR-9 up to 13 for VNTR-2 (Table 2). The percentage of sequence conservation of the repeats was above 90% for each VNTR locus.

Genogrouping of MLVA profiles

MLVA typing of 392 isolates resulted in 127 different MTs. An UPGMA clustering of the MLVA profiles revealed the existence of five MLVA genogroups (A, B, C, C1, and R) (Figure 1). Isolates within each group shared repeat numbers in at least two of the six loci (>33%). Naming of the MLVA genogroups was based on MLST and AFLP classifications (12, 34). The majority of isolates (74%) clustered similarly when typed by either MLVA, MLST, or AFLP (Figure 1). With MLVA, the majority of community survey isolates (12 of 17) and pigs (16 of 20) clustered in genogroup A. Isolates from poultry (13 of 13) and calf (19 of 19) clustered in genogroup B, clinical infection isolates (102 of 126) clustered in genogroups C and C1, and all but 1 hospital outbreak isolate (24 of 25) clustered in genogroup C1. The hospital survey isolates were proportionally represented among genogroups A (17 isolates), B (7 isolates), C (17 isolates), and C1 (24 of 68 isolates). Finally, a small number of isolates of miscellaneous origin clustered in genogroup R (Table 1). The UPGMA clustering of MLVA profiles confirmed clustering of isolates that belonged to the MLST-C1 group (12 isolates) into one MLVA-C1 group with the exception of a single hospital outbreak clone, NL-3-1 (MT-22) (Table 1 and Figure 1). In addition, three outbreaks—GR-3 (MT-5), NL-2-2 (MT-25), and US-1 (MT-1)—that did not cluster in the epidemic C1 genogroup by


MLVA MT
No. of strains
MLST ST
MLST group
AFLP group
VNTR -1
VNTR -2
VNTR -7
VNTR -8
VNTR -9
VNTR -10
C1
C

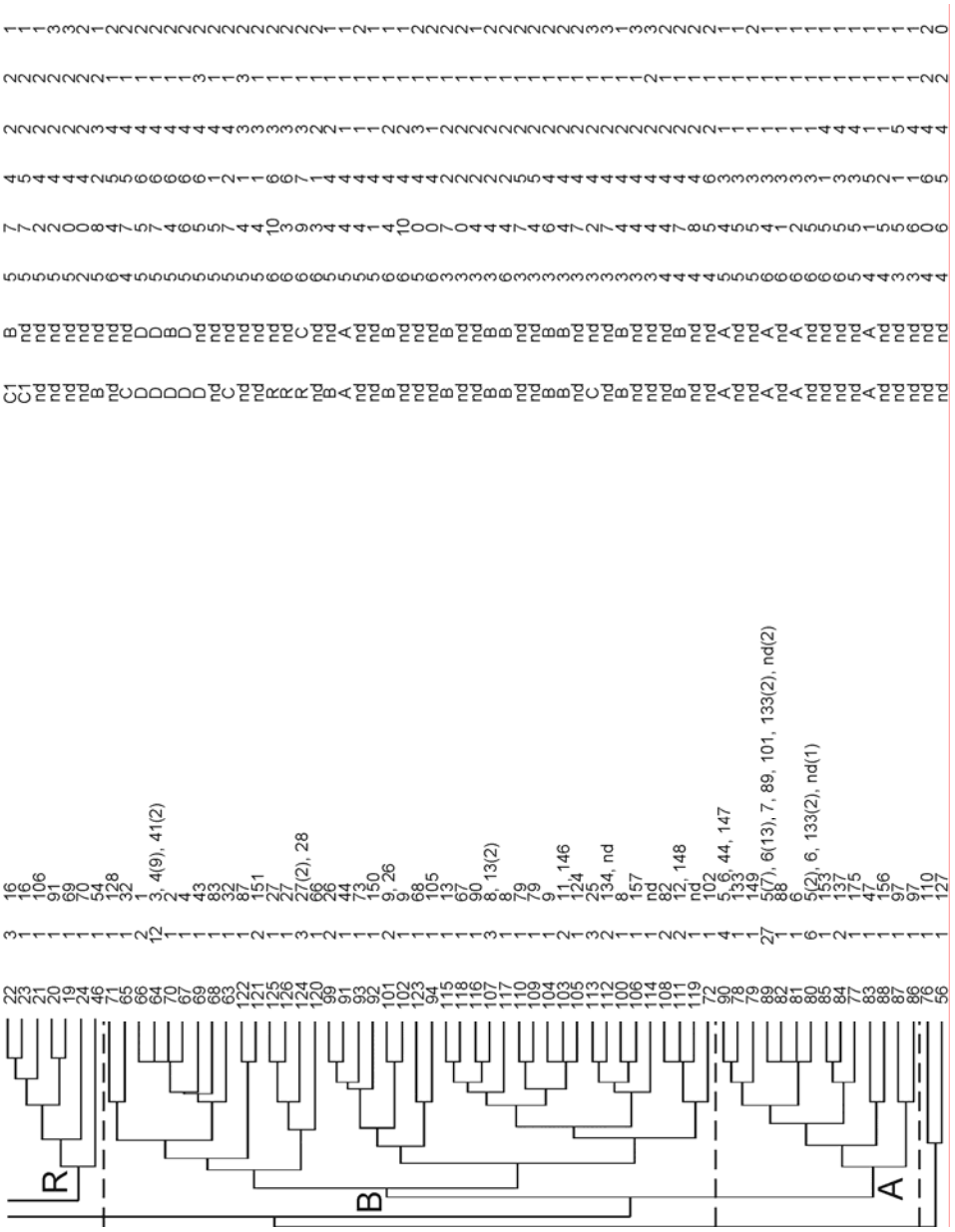


Figure. 1. UPGMA clustering of the 127 different MLVA profiles with a categorical similarity coefficient. For each MT, the number of isolates, MLST sequence types, MLST and AFLP genogroups, and MLVA profile are depicted. C1, epidemic isolates (24 of 25 isolates); C and C1, clinical infection (102 of 126 isolates); B, calf (19 of 19 isolates) and poultry (13 of 13 isolates); A, community survey (12 of 17 isolates) and pigs (16 of 20 isolates). A (17 isolates), B (7 isolates), C (17 isolates), and C1 (24 of 68 isolates), hospital survey; R, miscellaneous origin.

MLST (data not shown) grouped within the MLVA-C1 cluster. UPGMA clustering was used to assess the genetic relationship of MLVA profiles and to define and compare genogroups by MLST and AFLP. UPGMA was not suitable for obtaining insight in the evolutionary descent of *E. faecium* MTs. For this purpose, the eBURST algorithm was used. eBURST was originally developed for the analysis of large MLST data sets to reveal biologically meaningful clusters of sequence types and patterns of evolutionary descent from predicted founder types (7). As both MLST and MLVA profiles are based on a combination of numbers, eBURST should also be suitable for cluster analysis of MLVA profiles (Figure 2). In Figure 2, the dotted circle surrounds all MLVA profiles belonging to the MLVA-C1 genogroup based on UPGMA clustering except for MT-33, MT-34, MT-35, and MT-37, which are double-locus variants and therefore not connected to the MLVA-C1 complex. The eBURST clustering suggested that MT-1 is the primary founder of the other MLVA-C1 types. In the MT-1 group, 12 of the 25 documented hospital outbreak isolates, 36 clinical infection isolates, and 12 hospital survey isolates were found. These 60 isolates originated from geographically widespread regions (the United States, Europe, Israel, Tanzania, and Brazil).

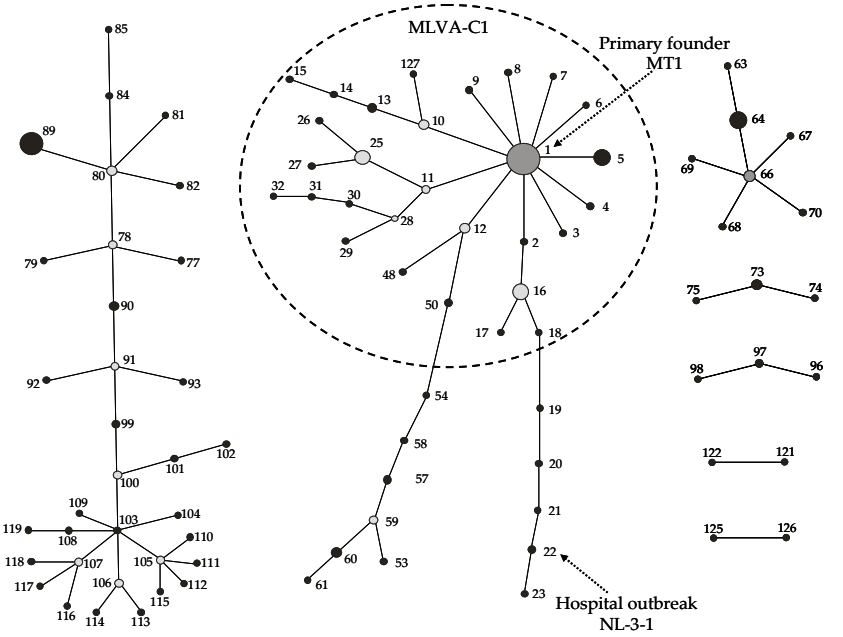


Figure 2. eBURST clustering of the 127 different MLVA profiles. In the eBURST algorithm, each MT is represented as a node (solid black dot). For clarity, only clusters or clonal complexes of related MTs are depicted. Dark-gray dot, primary founder. Light-gray dots, subgroups or secondary founders. The clinical relevant genogroup MLVA-C1 based on the UPGMA clustering is surrounded with a dotted circle.

Comparing the level of discrimination of MLVA, MLST, and AFLP

The discriminatory ability of MLVA, MLST, and AFLP was determined and compared by calculating the genetic diversity (*D*) with 95% CIs of 78 isolates typed by all three methods. MLVA showed the same level of discrimination as MLST and AFLP (Table 3). When the *D* values of different combinations of VNTR loci and the complete MLVA scheme of 291 isolates were compared to MLST, the genetic diversity of MLST and the complete MLVA were comparable. The *D* value of MLVA based on combinations of a limited number of VNTR loci was lower (Table 3). One exception was the combination of VNTR loci 1, 7, 8, and 10, in which the 95% CI of the genetic diversity of this profile just overlapped with that of MLST. Using MLST as a reference, sensitivities of individual VNTR loci and profiles of different VNTR loci combinations to identify isolates that belong to the MLST-C1 cluster ranged from 76% for typing based on VNTR-8 to 97% for MLVA of the complete set of loci. Remarkably, 97% of the MLST-C1 isolates appeared to have three repeats for VNTR-7. The specificity for VNTR-7 was the lowest 65%, but increased to 90% for MLVA on the complete set of loci and for MLVA with combinations of VNTR loci 7, 8, and 9 or VNTR-7, VNTR loci 8, 9, and 10 (Tables 3 and 4). The PPVs and NPVs to identify the MLST-C1 cluster were calculated for the complete MLVA profile and for a combination of either VNTR loci 7, 8, 9, and 10 or VNTR loci 7, 8, and 10. The PPV was 87% and the NPV was 97% for all three VNTR locus combinations analyzed (Table 4).

Stability of tandem repeats in outbreaks and in vitro

To analyze the stability of MLVA profiles, MLVA profiles of isolates recovered during two related outbreaks in the years 2000 to 2003 were determined. The collection comprised 32 isolates collected during hospital outbreak NL-1-1 and 18 isolates collected during hospital outbreak NL-2-1. With the exception of two isolates, all isolates from both outbreaks were shown to have MT-16. One patient carried the outbreak strain for more than 2 years. The MLVA profiles of the isolates recovered from this patient remained unchanged (data not shown). These data suggest that MLVA profiles are stable among strains that are recovered during a hospital outbreak. Two patients from outbreak NL-2-1 acquired colonization with a VREF belonging to MT-17, which is a single-locus variant of MT-16, in which VNTR-1 changed from five to six repeats. The stability of the MLVA profiles in vitro was determined by repeated subculturing of various isolates with a known MLVA profile; no change in MLVA profiles was observed in any case (data not shown).

Table 3. Comparison of Simpson's index of diversity (*D*) and 95% confidence interval (CI)

	Isolates typed by:			
	AFLP, MLST and MLVA[a]		MLST and MLVA[b]	
Typing Method	*D*	CI	*D*	CI
AFLP	0.94	0.90-0.97		
MLST	0.94	0.91-0.97	0.96	0.95-0.97
MLVA	0.93	0.90-0.97	0.95	0.92-0.97
VNTR loci 1-7-8-9-10			0.93	0.91-0.95
VNTR loci 1-7-8-10			0.91	0.89-0.93
VNTR loci 2-7-8-10			0.90	0.87-0.93
VNTR loci 7-8-9-10			0.91	0.89-0.93
VNTR loci 7-8-10			0.86	0.83-0.89
VNTR loci 1-8-10			0.90	0.88-0.92
VNTR loci 1-8-9			0.90	0.88-0.92
VNTR loci 2-7-10			0.86	0.82-0.90

[a]A total of 78 isolates were tested.

[b]A total of 291 isolates were tested.

Discussion

The availability of a fast, reproducible, cheap, and highly discriminatory bacterial typing method is essential for hospital epidemiology. MLVA is a typing technique that combines these characteristics and has been used to type and study the transmission of various bacterial species (16, 28). In this study, we have developed an MLVA typing scheme for *E. faecium* and compared its discriminatory ability to that of AFLP and MLST. The data presented here show that MLVA is extremely useful for studying the genetic relatedness of *E. faecium* isolates. Furthermore, MLVA can be used to study the local and global epidemiology of *E. faecium*.

The comparison of Simpson's index of diversity revealed that MLVA achieved the same degree of discrimination as MLST or AFLP. Moreover, UPGMA cluster analysis of MLVA profiles showed a degree of clustering of isolates in genogroups previously found by AFLP and MLST (12, 34). One specific MLVA genogroup designated MLVA-C1 harbored the majority of clinical isolates and all but one of the hospital outbreak-associated isolates. This genogroup was highly comparable to the MLST-C1 genogroup, which contained epidemic and clinical isolates (12). One hospital outbreak did not cluster in both the MLVA-C1 and the AFLP-C genogroup. Isolates from this outbreak clustered together with the other

Table 4. MLVA of 291 isolates to identify isolates belonging to the MLST-C1 group

Procedure (no. of repeats)	Result				Profiles indicative of MLST-C1[c]			
	Sensitivity (%)	Specificity (%)	PPV[a]	NPV[b]	VNTR-7	VNTR-8	VNTR-9	VNTR-10
Complete MLVA	97	90	87	97				
MLVA with VNTR loci 7-8-9-10	97	90	87	97	3	3	2	3
					3	2	2	3
					3	3	2	2
					4	3	2	3
					3	3	3	3
					3	3	1	3
					3	4	3	3
					3	3	3	1
					4	3	3	3
					4	2	3	3
					3	1	2	3
					3	4	1	3
MLVA with VNTR loci 7-8-10	97	90	86	97	3	3		3
					3	2		3
					3	3		2
					4	3		3
					3	4		3
					4	2		3
					3	3		1
VNTR-1 (5)	82	66	63	84				
VNTR-2 (6, 7)	92	68	66	93				
VNTR-7 (3)	96	65	65	96				
VNTR-8 (3)	75	82	74	83				
VNTR-9 (2)	79	83	76	85				
VNTR-10 (3)	92	66	65	93				

[a] PPVs are the number of MLST-C1 and MLVA-C1 isolates divided by the number of MLST-C1 and MLVA-C1 plus MLST-nonC1 and MLVA-C1 isolates.

[b] NPVs are the number of MLST-nonC1 and MLVA-nonC1 negative isolates divided by the number of MLST-nonC1 and MLVA-nonC1 plus MLST-C1 and MLVA-nonC1 isolates.

[c] Values are the number of repeats.

outbreak-associated isolates only by MLST. In contrast, three other hospital outbreak isolates with three different MLST sequence types that clustered outside the MLST-C1 group did group within MLVA-C1. eBURST confirmed the grouping of clinical relevant isolates in the MLVA-C1 cluster and revealed that MT-1 was the primary founder of this cluster. The existence of a cluster of clinical and epidemic *E. faecium* isolates was also reported previously (12, 18, 34).

We determined the sensitivity, specificity, PPV, and NPV of MLVA based on combinations of various VNTR loci to identify isolates that belong to the MLST-C1 cluster. It appeared that a single VNTR locus, VNTR-7, yielded the same sensitivity (97%) in identifying genotypes belonging to MLST-C1 as the complete MLVA profile, yet specificity and PPV were rather low (65%). Addition of the VNTR loci 8, 9, and 10 increased specificity to 90% and PPV to 87%, comparable

to the complete MLVA profile. Consequently, for rapid screening to identify epidemic isolates, a multistage approach can be used, starting with a single PCR to determine the number of repeats in VNTR-7. Subsequent PCRs with VNTR loci 8, 9, and 10 can confirm the potential epidemic nature of the isolates, when the single PCR revealed three repeats for VNTR-7. However, for library typing and study of the epidemiology of *E. faecium*, it is recommended that MTs be assigned that based on all six VNTR loci, since the index of diversity decreased strongly when loci were excluded from the full MLVA profile.

MLVA of multiple isolates from two hospital outbreaks (NL-1-1 and NL-2-1) (19, 32) were used to analyze the stability of the tandem repeats and thus the MLVA profiles; such analysis is a prerequisite for identifying and studying hospital outbreaks. The first isolates from these outbreaks date from 2000 and were found in patients from the nephrology departments of both hospitals. Most of the patients were hemodialysis patients who came to the hospital on a regular basis and were often treated with antibiotics, including vancomycin. From the 50 isolates collected during a 3-years period, only 2 isolates isolated from two different patients were found to have a divergent MLVA profile. However, this was only due to the addition of a single repeat in one of the six VNTR loci. This means that MLVA profiles generally remain unchanged during hospital outbreaks, demonstrating that MLVA can be used to study local outbreaks.

The MLVA method described in this study is much faster and cheaper than MLST, because it is a PCR-based method that utilizes simple agarose gels for analysis. AFLP is also considered a fast typing method, but the reproducibility and portability of AFLP are problematic. The fact that this MLVA uses agarose gels to detect and size amplicons also makes it an attractive typing method for use in local laboratories to determine patient-to-patient transmission and to identify potential epidemic isolates. MLVA profiles can easily be stored in a database, facilitating the exchange of MLVA typing data through a Web-based database. Therefore, an Internet site (http://www.mlva.umcutrecht.nl) has been developed for the submission of MLVA profiles to assign the MTs. Furthermore, a strain database is available on the Web site, which contains MTs as well as strain characteristics like MLST sequence type, isolate source, and country of isolate origin.

In the *E. faecium* MLVA scheme, tandem repeat loci were chosen that, according to the partially annotated *E. faecium* genome sequence (http://genome.ornl.gov/microbial/efae/), were not located within known open reading frames. Therefore, we assume that changes in repeat numbers are not the result of selective pressure, which means that MLVA data can also be used to study the phylogeny of *E. faecium*. However, since the *E. faecium* genome

sequence is not yet annotated completely, we cannot exclude that some of the loci are located within coding or regulatory regions.

In conclusion, we developed a fast, reproducible, cheap, and portable typing method that can be used as a tool to study the epidemiology of *E. faecium* in general and to rapidly detect potential epidemic isolates. We suggest using MLVA as an initial method to screen and type *E. faecium* in hospital laboratories. Subsequently, MLVA profiles could be added to a Web-based database for international comparison, and representative isolates could be subjected to MLST to gain insight into the global epidemiology of particular MLVA profiles.

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Chapter 4

Comparison of Multiple-Locus Variable-Number Tandem Repeat Analysis and Pulsed-Field Gel Electrophoresis in a Setting of Polyclonal Endemicity of Vancomycin-Resistant *Enterococcus faecium*.

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Abstract

Genotyping of vancomycin-resistant *Enterococcus faecium* (VREF) isolates is important to study its epidemiology in hospitals. Although pulsed-field gel electrophoresis (PFGE) is considered the "gold standard" genotyping method for VREF, it is labor intensive and lacks standardized methods and interpretative criteria, thereby hampering interlaboratory data exchange. Multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA) is a rapid, PCR-based typing scheme that yields an unambiguous numerical result suitable for data exchange between laboratories. In order to assess whether MLVA could replace PFGE, we compared the typeability, discriminatory power, concordance and costs of these methods for VREF isolates obtained from patients, environmental samples and health care worker (HCW) hands in a medical ICU where VREF are endemic. Over a 58-day period, 393 VREF isolates were collected from cultures of patient rectal swabs (77), the environment (268) and HCW hands (48). For PFGE, 358 (91.1%) isolates were typeable, yielding 19 PFGE types (> 6 bands different) and 24 subtypes (1-3 bands different). MLVA typed 391 (99.5%) isolates, generating 11 genotypes. The discriminatory power of PFGE subtypes was 83%, compared to 68% for MLVA. Concordance between the 2 methods based on matched or mismatched MLVA-types and PFGE-types or -subtypes was 67.5% and 82.8%, respectively. By PFGE, 13 isolates could be genotyped in 3 days at an estimated cost of $10 per isolate; MLVA genotyped 94 isolates in 2 days at $11 per isolate. PFGE and MLVA were highly concordant in assigning genotypes to nosocomial VREF isolates. MLVA was faster but PFGE subtyping was more discriminatory.

Introduction

Since 1989, vancomycin-resistant *Enterococcus faecium* (VREF) have emerged as nosocomial pathogens in the United States (U.S.) (3), especially in immunocompromised patients (14). VREF are now endemic in many hospitals (3). Recently multilocus sequence typing (MLST)-based molecular epidemiological studies of both human and animal derived vancomycin-resistant and vancomycin-susceptible *E. faecium* isolates revealed the existence of host-specific genogroups. A specific genogroup, labeled clonal complex-17 (CC17), is associated with nosocomial outbreaks and infections on five continents and characterized by ampicillin- and quinolone-resistance and the presence of the *esp* virulence gene (10,11,20,21).

Genotyping of isolates is important to study the epidemiology of VREF in hospitals. So far, pulsed-field gel electrophoresis (PFGE) has been considered the

"gold standard" method for genotyping of enterococci (9,13,15,17,19). However, PFGE is labor-intensive, and lacks standardized methods and strict criteria for interpretation of banding patterns, thereby hampering interlaboratory data exchange. Multiple-locus variable-number tandem repeat analysis (MLVA) is based on differences in the variable number of tandem repeats (VNTR) on multiple loci on the chromosomes of bacteria, which can rapidly be detected by PCRs with specific primers based on the flanking regions of the tandem repeats. Because MLVA types are discriminated by gain or loss of discrete repeats, this method yields an unambiguous numerical result suitable for data exchange between laboratories via internet (18).

To determine whether PFGE can be replaced by MLVA, we compared typeability, discriminatory power, concordance, cost and turn around time of these two methods for VREF isolates obtained from patients, environmental samples and health care worker (HCW) hands in a medical ICU where VREF colonization is endemic.

Materials and methods

Bacterial isolates

Between March and May 2001 VREF were isolated from patient rectal swab specimens, samples of healthcare worker hands and of swab samples of environmental sites in the 21-bed medical intensive care unit (MICU) at Rush University Medical Center, Chicago, Illinois, USA. This 58 day period was part of a larger intervention study to reduce the spread of VREF and of other species of vancomycin-resistant enterococci (5).

PFGE

PFGE typing of *Sma*I-digested DNA was performed as described by Kim et al. (7). The assignment of PFGE-types (PT) was adapted from the Tenover criteria (17). Isolates that differed by ≤ 6 bands were assigned the same PT and isolates that differed by ≥ 7 bands were considered different types. Within each PT, isolates that differed by 1-3 bands were assigned the same subtype (PST).

MLVA typing

MLVA typing was performed as described previously (18), with the following minor modifications. PCR on *ddl*, VNTR-1, -7, -8, -9 and -10 was performed using HotStarTaq master mix (Qiagen inc., Valencia, CA, USA), while PuRe Taq Ready-To-Go PCR beads (GE healthcare Bio-sciences AB, Uppsala, Sweden) were used for VNTR-2. The PuRe Taq Ready-To-Go PCR beads were dissolved in 20 µl MilliQ water including 10 pmol of both the forward and reverse primer for VNTR-2 and finally 5 µl of lysate was added. Furthermore, PCR programs for

VNTR-8 and VNTR-9 were similar to VNTR-7 and VNTR-10, while for VNTR-2 the extension time was prolonged to 2 minutes, instead of 30 s, at 72°C.

Identification of CC17 specific MLVA types was performed by comparing the MLVA profiles obtained with the previously described different repeat combinations for VNTR-7, -8 and -10, which have been shown to have a positive predictive value of 87% and a specificity of 90% for CC17, formerly designated MLST-C1 (18).

Esp PCR

All strains were screened for the *esp* gene (as a marker gene for the presence of the putative pathogenicity island (10,20)) by PCR with the primers *esp*$_{fm}$.14F and *esp*$_{fm}$.12R as described previously (12).

Statistics

Difference in typeability for MLVA and PFGE was analyzed by Chi-square test. To compare the discriminatory abilities of PFGE and MLVA, Simpson's index of diversity (*D*) and 95% confidence intervals (CI) were calculated for the number of isolates typed by both methods according to formulas previously described (4,6) using EpiCompare (version 0.99; Ridom GmbH, Würzburg, Germany). The concordance between MLVA type and PFGE types and subtypes was calculated using EpiCompare (version 0.99; Ridom GmbH). For this analysis all possible pairs of isolates were cross-classified on the basis of matched or mismatched types according to the method described by Robinson *et al.* (16). The significance of the concordance was not estimated because the pairs on which the analysis was based are not independent (16).

Table 1. Characteristics of PFGE and MLVA

Characteristic	PFGE n=393	PFGE-Subtype	MLVA n=393
Typeability	358 (91.1%)		391 (99.5%)
Discriminatory power	0.4	0.83	0.68
95% Confidence interval	0.33- 0.46	0.81 - 0.85	0.65 - 0.71
Duration of procedure[a]	3 days		2 days
Duration of type assignment[a]	Hours		Hours
Estimated cost per isolate[b]	$10		$11

[a]Duration of procedure and duration of type assignment are estimates for a single PFGE gel on which 13 isolates and 2 controls are analyzed, and a single MLVA plate on which 94 isolates and 2 controls are analyzed

[b]Cost per isolate tested, including materials, labor and equipment depreciation, in 2005 U.S. dollars.

Abbreviations: PFGE, pulsed-field gel electrophoresis; MLVA, multiple-locus variable-number tandem repeat analysis.

Results

Patients and bacterial data

The VREF isolates analyzed were from a larger data set of vancomycin-resistant *E. faecium* and *E. faecalis* isolates identified during the baseline, non-intervention period of a previously published study (5). A total of 146 patients were admitted to the MICU during the baseline study period and 393 VREF isolates, all ampicillin-resistant, were identified in 77 rectal swabs from 33 patients, 268 environmental samples and 48 samples of HCW hands. Four patients in the MICU were colonized with vancomycin-resistant enterococci at the start of the study period, while 29 (19.9%) patients were colonized on admission. Of the remaining 113 patients, 16 (14.2%) acquired vancomycin-resistant enterococcal colonization during their stay in MICU (5).

Table 2. Distribution of MLVA types, including the MLVA profiles among the isolates

MLVA type	MLVA profile[a]						No. of isolates	CC17 specific MTs[b]	%
	VNTR-1	VNTR-2	VNTR-7	VNTR-8	VNTR-9	VNTR-10			
1	5	7	3	3	2	3	34	+	7.7
2	5	6	3	3	2	3	177	+	43.5
5	5	7	3	2	2	3	11	+	2.3
9	5	3	3	3	2	3	1	+	0.3
163	5	5	3	3	2	3	1	+	0.3
206	5	6	3	3	1	3	140	+	34.5
208	5	8	3	2	1	1	44	-	10.5
209	5	8	3	3	2	3	1	+	0.3
211	5	6	3	2	1	1	1	-	0.3
212	5	4	3	3	1	3	1	+	0.3
213	4	6	3	3	2	3	1	+	0.3

[a]Numeric values in the table refer to the number of repeats for each VNTR locus.

[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% (18).

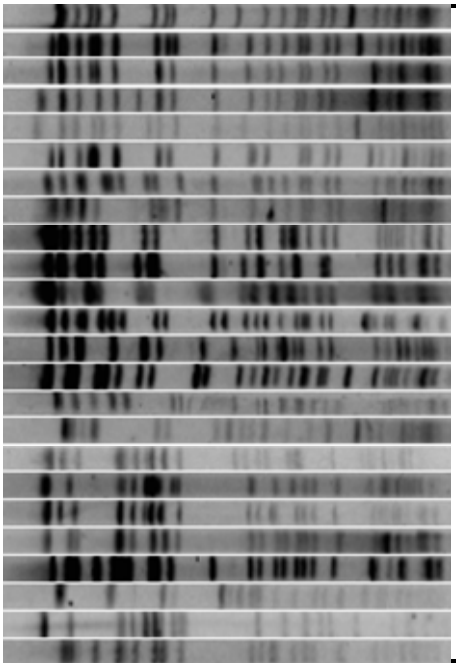
Abbreviations: MLVA, multiple-locus variable-number tandem repeat analysis; VNTR, variable number tandem repeat.

VREF genotyping

Of 393 VREF isolates, 358 (91.1%) yielded interpretable PFGE banding patterns the first time they were tested (Table 1), resulting in 19 PTs and 24 PSTs (Figure 1). PT-1 was the predominant type (275/358; 76.8%). Within PT-1, 4 PSTs were found of which PST-1B and PST-1C accounted for 36.0% (99/275) and 31.2% (86/275) of the PT-1 isolates, respectively. PT-6 revealed 3 subtypes.

MLVA generated complete MLVA profiles in 391 of 393 VREF (99.5%) (Table 1), representing 11 MLVA types (MTs) (Table 2). MT-2 was the predominant type, accounting for 43.5% of the isolates (170/391). MT-206 and MT-1, which are single locus variants of MT-2, were found among 135/391 (34.5%) and 30/391 (7.7%) of the isolates, respectively. MT-208 accounted for 10.5% of the isolates (41/391).

All but 19 MT-1 isolates contained the *esp* gene, representing 374/393 (95.2%) of the isolates.



PFGE subtype (PST)	No. of isolates	%
1A	47	13.1
1B	99	27.7
1C	86	24
1D	43	12
2	1	0.3
3	1	0.3
4	2	0.6
5	1	0.3
6A	30	8.4
6B	9	2.5
6C	1	0.3
7	4	1.1
8	6	1.7
9	3	0.8
10	1	0.3
11	3	0.8
12	1	0.3
13	4	1.1
14	2	0.6
15	9	2.5
16	2	0.6
17	1	0.3
18	1	0.3
19	1	0.3

Figure 1. Overview of representative banding patterns for 24 pulsed-field gel electrophoresis-subtypes, with corresponding numbers of isolates and the percentage of the total number of isolates. PFGE types 1 and 6 comprised 76.8% and 11.2%, respectively of all PFGE types identified.

Performance of MLVA compared to PFGE

Typeability of MLVA (99.5%) was significantly higher than PFGE (91.1%) (p<0.01) (Table 1). The discriminatory ability of PFGE-types, -subtypes and MLVA was determined and compared by calculating the discriminatory power (D) with 95% CIs of 356 isolates typed with both methods. MLVA had a higher

level of discrimination compared to PFGE-types, but lower compared to PFGE-subtypes (Table 1). The total time required to complete one PFGE gel with 13 samples, including type assignment was approximately 3 days, while approximately 2 days was required to determine MLVA profiles of 94 samples. The estimated cost per isolate tested, including materials, labor and equipment depreciation, was similar for the 2 methods (Table 1).

Cross-classifying the isolates based on matched or mismatched MT and PT or PST showed that the concordance of the typing schemes was 67.5% and 82.8%, respectively (Table 3). When compared to PTs, MTs were more predictive for the PTs than vice versa. Ninety-four percent (18953/20211) of all isolate pairs sharing identical MTs also had identical PTs, while 50% (18953/38214) of the pairs with identical PTs shared identical MTs, illustrating that MLVA provided a more discriminatory dataset than PFGE patterns classified into PTs. In contrast, PSTs were more predictive for MTs, than vice versa. Ninety-three percent (10133/10906) of all pairs sharing identical PSTs also had identical MTs, while 50% (10133/20211) of the pairs with identical MTs shared identical PSTs (Table 3). This can be explained by the greater diversity in PFGE-subtype classification.

An advantage of MLVA typing is that it can be used to identify isolates belonging to CC17. Comparison of the obtained MLVA profiles with the previously described CC17 specific repeat combinations for VNTR-7, -8 and -10 (18) revealed that except for MT-208 and MT-211, all MLVA profiles belonged to CC17, representing 349/391 isolates (89.3%).

Table 3. Cross-classification of all possible pairs of isolates based on matched or mismatched MTs and PTs or PSTs

		PFGE-type			PFGE-subtype		
		Match	Mismatch	Sum	Match	Mismatch	Sum
MLVA	Match	18953	1258	20211	10133	10078	20211
	Mismatch	19261	23718	42979	773	42206	42979
	Sum	38214	24976	63190	10906	52284	63190
		Concordance = 67.5%			Concordance = 82.8%		

[a]All pairwise comparisons that were undistinguishable by both MLVA and PFGE (match-match) or were considered different by MLVA and PFGE (mismatch- mismatch) are in concordance.

Abbreviations: PFGE, pulsed-field gel electrophoresis; MLVA, multiple-locus variable-number tandem repeat analysis; MT, MLVA type; PT, PFGE type; PST, PFGE subtype.

Distribution of PSTs among MTs

The three most prevalent MTs (MT-2, MT-206, and MT-208) were predominantly linked to specific PTs: MT-2 and MT-206 to PT-1, 148/153 and 122/123, respectively, and MT-208 to PT-6 (36/37) (Table 4). Linkage to a specific PT was not found for MT-1. At the start of the study period MT-2 and MT-208

were represented by a single PST; PST-1A in MT-2 and PST-6A in MT-208, while MT-206 was represented by two PSTs, PST-1C and PST-1D, of which PST-1C clearly dominated. Over time the dominant PST in MT-2 changed from PST-1A to PST-1B. In MT-206 the proportion of PST-1C first decreased over time in favor of PST-1D, while at the end of the study period MT-206 almost disappeared. Finally, PST-6A was partly replaced by PST-6B among MT-208 isolates.

Discussion

This study reveals that MLVA typing may serve as a faster, more standardized, but less discriminatory alternative to PFGE typing to study the genetic relatedness of VREF within hospitals. Results of PFGE and MLVA typing were highly concordant. The typeability for MLVA, however, was significantly higher than for PFGE. Although PFGE has been described as a highly reproducible method for *E. faecium* (19) the feasibility of generating banding patterns may differ substantially among isolates, and it is not always clear what causes the diversity of banding pattern quality. Use of a band-based cluster tool, like the Dice algorithm found in some software packages, abrogates some of these difficulties, but assignment of bands is still subject to personal interpretation and an important cause of lack of reproducibility. Strict standardized procedures and techniques are necessary for interlaboratory comparison (8). In contrast, MLVA is a highly reproducible method (18) with the advantage that in the PCR single bands are produced. The translation from band size to number of repeats can easily be read from the MLVA website (www.mlva.umcutrecht.nl), where also the type assignment can be made and compared to a database, including isolates from different continents.

The stability of PFGE banding patterns in *E. faecium* has been studied by Morrison *et al*. (13). A single colony was subcultured repeatedly and a large degree of DNA banding pattern polymorphism was observed, which the authors hypothesized was due to mobile element-induced genomic rearrangement. In contrast, in a study by Bonten *et al.* little genetic variation was found among isolates cultured from long-term colonized patients (2). Within the patient group in the current study there are several examples where the environmental and rectal samples from one patient collected on one day showed different PSTs, but the same MT (data not shown). Among MT-2, -206 and -208 isolates a shift in PSTs was observed over time, which can be explained by genetic rearrangements. Therefore it could be argued that genetic diversity inferred from PFGE banding patterns, especially when stringent criteria like the PFGE subtype criteria in this study are used, is too high to recognize clones or events of cross-transmission in a setting of endemicity. MT-1 did not correlate to a single PT. It is possible that

Table 4. Distribution of PFGE-subtypes among MLVA types[a]

MLVA	PFGE-subtypes																							
MT	1A	1B	1C	1D	2	3	4	5	6A	6B	6C	7	8	9	10	11	12	13	14	15	16	17	18	19	Total
1						1							6			3	1	4	2	8		1	1	1	28
2	47	95	1	5				1	2											1	1				153
5							2					4		3											9
9					1																				1
163		1																							1
206		1	84	37																	1				123
209		1																							1
212				1																					1
213			1																						1
208									26	9	1				1										37
211									1																1
Total	47	98	86	43	1	1	2	1	29	9	1	4	6	3	1	3	1	4	2	9	2	1	1	1	356

[a] MLVA types predicted to belong to CC17 are indicated in bold. Numbers in the body of the table indicate the number of isolates belonging to each group.

Abbreviations: PFGE, pulsed-field gel electrophoresis; MLVA, multiple-locus variable-number tandem repeat analysis.

genome rearrangements account for this observation. However, previous MLST-based studies demonstrated that MT-1 is polyclonal (18). Our observation that, in contrast to the other MTs, *esp* is present in only a subset of MT-1 underscores polyclonality of MT-1.

Based on MLVA profiles, ampicillin resistance and the presence of *esp*, >85% of the isolates were identified to belong to CC17. However, two CC17 characteristic features, ampicillin resistance and the presence of the *esp* gene, were also found among the MT-208 and MT-211 isolates. This suggests that in addition to previously described MLVA profiles (18), other MLVA profiles found within this cluster may also be linked to CC17. Multilocus sequence typing of representatives of MT-208 and MT-211 will ultimately show whether or not isolates with these MLVA profiles group within CC17. To our knowledge there are no prior studies that describe PFGE banding patterns' linkage to CC17.

In the current study the discriminatory power of MLVA was lower (0.68) than reported previously (1,18). This is due to the fact that we analyzed isolates that were collected over a relatively short period from patients on a single hospital ward where VREF was endemic, so that highly related clones were to be expected. An earlier study in which the discriminatory power of MLVA was found to be 0.95 (18) evaluated a more genetically diverse strain collection that included isolates from various ecological niches such as the community, animals, the environment and hospitals, and from different geographic locations. A more recent investigation that found the discriminatory power of MLVA to be 0.846 analyzed VREF isolated over a 2 year period from patients housed on different

hospital wards (1); the genetic diversity of these isolates would also be expected to be greater than the diversity in the current strain set.

In conclusion, MLVA is highly congruent to PFGE but faster. It provides less ambiguous typing data that are easier to store in databases and to exchange between laboratories. MLVA is able to link isolates to CC17, which is not possible with PFGE. However, analysis of PFGE subtypes has a greater ability to discriminate among VREF strains than does MLVA. Both MLVA and PFGE are useful methods for determining clonal spread of VREF in an MICU setting of high-level, polyclonal endemicity.

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Chapter 5

A Novel Putative Enterococcal Pathogenicity Island Linked to the *esp* Virulence Gene of *Enterococcus faecium* and Associated with Epidemicity

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Abstract

Enterococcus faecalis harbors a virulence-associated surface protein encoded by the *esp* gene. This gene has been shown to be part of a 150-kb putative pathogenicity island. A gene similar to *esp* has recently been found in *Enterococcus faecium* isolates recovered from hospitalized patients. In the present study we analyzed the polymorphism in the *esp* gene of *E. faecium*, and we investigated the association of *esp* with neighboring chromosomal genes. The *esp* gene showed considerable sequence heterogeneity in the regions encoding the nonrepeat N- and C-terminal domains of the Esp protein as well as differences in the number of repeats. DNA sequencing of chromosomal regions flanking the *esp* gene of *E. faecium* revealed seven open reading frames, representing putative genes implicated in virulence, regulation of transcription, and antibiotic resistance. These flanking regions were invariably associated with the presence or absence of the *esp* gene in *E. faecium*, indicating that *esp* in *E. faecium* is part of a distinct genetic element. Because of the presence of virulence genes in this gene cluster, the lower G+C content relative to that of the genome, and the presence of *esp* in *E. faecium* isolates associated with nosocomial outbreaks and clinically documented infections, we conclude that this genetic element constitutes a putative pathogenicity island, the first one described in *E. faecium*. Except for the presence of *esp* and *araC*, this pathogenicity island is completely different from the *esp*-containing pathogenicity island previously disclosed in *E. faecalis.*

Introduction

Enterococci are common inhabitants of the gastrointestinal tracts of humans and animals, and although they have been recognized as pathogens able to cause endocarditis, they were generally considered second-rate pathogens. Recent estimates, however, indicate that enterococci are now among the leading causes of nosocomial infections (57). Of all enterococcal species, *Enterococcus faecalis* accounted for the most infections in humans (26). However, during the past decade, the incidence of bloodstream infections caused by *Enterococcus faecium* increased, an increase which has been linked to the emergence of antibiotic resistance in this species (26, 40).

Little is known about virulence determinants in *E. faecium* (20). Recently, however, three potential virulence genes, *esp*, *hyl*, and *acm*, have been described for *E. faecium*. They were all found more frequently in clinical isolates than in fecal isolates or nonhuman isolates (13, 41, 44, 65).

Of these three putative virulence genes, only the *esp* gene is also found in *E. faecalis* (51). The Esp protein in *E. faecalis* is expressed as a large surface-exposed

protein with a molecular mass of approximately 202 kDa. In *E. faecalis*, Esp is thought to be an adhesin contributing to colonization of urinary tract epithelial cells and biofilm formation (50, 59). Although detailed experimental evidence is not yet available, the higher prevalence of the *E. faecium esp* gene in clinical isolates suggests a role of Esp in the pathogenesis of *E. faecium* infections (3, 7, 12, 13, 30, 65, 68). Furthermore, the presence of the *esp* gene in *E. faecium* was also strongly associated with hospital outbreaks of vancomycin-resistant *E. faecium*, suggesting a role for Esp in nosocomial transmission (65).

Recently, the *esp* gene of *E. faecium* strain P61 was cloned and sequenced (13). Analysis of the sequence revealed that the enterococcal Esp (13, 51) belongs to a family of gram-positive surface-exposed proteins with repetitive structures such as the alpha C (38) and Rib (55) proteins of *Streptococcus agalactiae*, the R28 protein of *Streptococcus pyogenes* (54), and the Bap protein of *Staphylococcus aureus* (8), all of which are involved in virulence and in conferring protective immunity. Sequence similarity between these surface proteins is found predominantly in the repeat regions.

In *E. faecalis*, the *esp* gene is contained on a large (150-kb) genetic element (49). This element has all the characteristics of a pathogenicity island (PAI), with a GC content of 32.2%, which is significantly different from that of the rest of the *E. faecalis* chromosome, and the presence of genes encoding transposases, transcriptional regulators, and virulence determinants.

In this study we demonstrate considerable sequence heterogeneity among the *E. faecium esp* genes of various isolates. We also show that *E. faecium esp* is contained on a putative PAI and that the presence of this putative PAI is associated with nosocomial outbreaks of *E. faecium*.

(Part of this study was presented as a poster at the 42nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, Calif., 27 to 30 September 2002 [abstr. B-803].)

Materials and methods

Bacterial strains

E. faecium isolate E300 from hospital outbreak US-1 (11, 65) was used to clone and sequence the *esp* gene and the DNA region encompassing the putative PAI. *E. faecium* isolate E734 from hospital outbreak NL-1-1 (64, 65) and strain E470 from hospital outbreak NL-3-1 (58, 65) were used to determine sequence heterogeneity in the N- and C-terminal domains of the *esp* gene. Sequencing of the frameshift mutation at positions 12830 to 12832 and the stop codon at position 13719, originally found in strain E300, was performed for isolates E155 from outbreak US-2-6 (5, 65) and E734 from outbreak NL-1-1 (64, 65). Bacteria were grown on

Table 1. Oligonucleotides used in this study.

Name	Sequence	Strand	Start position	Target or function
esp $_{fs}$1R	5'-ACT ATC AAC CTC TCC TGT TTT AG	-	5616[a]	sequencing of the *esp* gene
esp $_{fs}$2R	5'-GAA GAG ACT TCT TCC TCT TTT C	-	5750[a]	sequencing of the *esp* gene
esp $_{fs}$3R	5'-TTC GGC GCT TTT TTA TC	-	5273[a]	sequencing of the *esp* gene; amplification C-repeat region
esp $_{fs}$4F	5'-GGA ACG CCT TGG TAT G	+	1000[a]	sequencing of the *esp* gene
esp $_{fs}$4R	5'-GAA TAT GTC ACT ACA ACC GTA C	-	3254[a]	sequencing of the *esp* gene
esp $_{fs}$5F	5'-GGA AAC CTG AAT TAG AAG AAG	+	1490[a]	sequencing of the *esp* gene
esp $_{fs}$5R	5'-TAC TGC TAA ATC GGT CGT G	-	2295[a]	sequencing of the *esp* gene
esp $_{fs}$6F	5'-ACG TGG ATG TAG AGT TTG C	+	1973[a]	sequencing of the *esp* gene
esp $_{fs}$6R	5'-CCG CTT TTG GTG ATT C	-	1798[a]	sequencing of the *esp* gene
esp $_{fs}$7F	5'-CGA CCG ATT TAG CAG TAA C	+	2279[a]	sequencing of the *esp* gene; amplification A-repeat region
esp $_{fs}$7R	5'-CCG CTG CTT TCA TTT C	-	1309[a]	sequencing of the *esp* gene
esp $_{fs}$8F	5'-GGT AGA GGT TGT TAT TTC TGT AGT AG	+	5233[a]	sequencing of the *esp* gene
esp $_{fs}$8R	5'-TAT AGA AAT CAT CTT GAT CTG TC	-	817[a]	sequencing of the *esp* gene
esp $_{fs}$9F	5'-AAA AGG TAC GGT TGT AGT GAC	+	3228[a]	sequencing of the *esp* gene; PCR
esp $_{fs}$9R	5'-TTC TTC GTA TAT CCC GG	-	305[a]	sequencing of the *esp* gene
esp $_{fs}$10F	5'-GAA ACA ACT GAT ACA CAA ACT G	+	610[a]	sequencing of the *esp* gene
esp $_{fs}$10R	5'-CAG TTT GTG TAT CAG TTG TTT C	-	631[a]	sequencing of the *esp* gene
esp $_{fm}$1F	5'-ATA ACT ATT AAG GGA GTT GAT TTG	+	3063[b]	sequencing of the *esp* gene
esp $_{fm}$2F	5'-TGG TTA GCA AGA ATA ATA AGA GAG	+	3092[b]	sequencing of the *esp* gene
esp $_{fm}$3R	5'-GGA CTT GCA TTA GCA AAA TC	-	4277[b]	sequencing of the *esp* gene
esp $_{fm}$4F	5'-AAG TAG AGG TTA CTA TTT CTG TAG AAG	+	8288[b]	sequencing of the *esp* gene
esp $_{fm}$5R	5'-CAG CTG CGC TAA CAT CTA C	-	6769[b]	amplification A-repeat region
esp $_{fm}$5F	5'-AAA GAA GAT TTA CCA AAA GAT ACT AAG	+	6706[b]	amplification C-repeat region
esp $_{fm}$4R	5'-AAT ACT CTC TTA TTA TTC TTG CTA ACC	-	3119[b]	inverse PCR, cloning and sequencing of PAI
nox 1F	5'-GTA ATT ATT GGA TCA AAC CAT TC	+	8939[b]	inverse PCR, cloning and sequencing of PAI
nox 2R	5'-GAA TGG TTT GAT CCA ATA ATT AC	-	8961[b]	sequencing of PAI; PCR
PAI-1R	5'-ACG TTC ATG TAT GGG AAA G	-	13814[b]	sequencing of PAI
PAI-2F	5'-GTA TTA GCG GTG TTC AAA ATG	+	49[b]	sequencing of PAI; PCR
PAI-2R	5'-TTC CTC TGT CAA AAT AAG CTA AC	-	755[b]	sequencing of PAI
PAI-3R	5'-CGA TAG GTG ACA GAA CTC ATA AC	-	14575[b]	sequencing of PAI; PCR
PAI-4R	5'-GTT CCA AAA AGG CTG ATA ATC	-	14059[b]	sequencing of PAI
PAI-5F	5'-CCT TGT TCC AGT CCC C	+	14524[b]	sequencing of PAI
PAI-5R	5'-AAA ATC AAG CCG CCA AG	-	13891[b]	sequencing of PAI
PAI-6F	5'-GAA GAA GGA ATT TGA AGT CAC	+	2697[b]	sequencing of PAI
PAI-6R	5'-CTA ATG ATC GTG TAG CTA AGA AC	-	13385[b]	sequencing of PAI
PAI-7F	5'-CGG ATC ATA ATA ATT ATT GTC TTT G	+	587[b]	sequencing of PAI
PAI-7R	5'-GAT ATT TGT CAA TCA AAG GTT G	-	12937[b]	sequencing of PAI; PCR
PAI-8R	5'-TTT AGA AGT CGC TTT GCC	-	12492[b]	sequencing of PAI
PAI-9R	5'-ATC AAA GGT CTA AGA ATC CAA C	-	11891[b]	sequencing of PAI
PAI-10R	5'-CAT AGG TTT TAA TTA ATT CAT TTA GC	-	11396[b]	sequencing of PAI
PAI-11R	5'-CGC AGA CTC ACC AAT TTT C	-	10987[b]	sequencing of PAI
PAI-12R	5'-CAG TCG TCT CGG TTC TTT C	-	10511[b]	sequencing of PAI
PAI-13R	5'-CAA AGC TAA TTC TTA ATT TTA CAC G	-	10067[b]	sequencing of PAI
PAI-14R	5'-CTT ATT ATT CTT GCT AAC CAT TAT TC	-	3111[b]	sequencing of PAI
PAI-15R	5'-ATT GGA GTT ATC AAC ATT TTT TC	-	2613[b]	sequencing of PAI
PAI-16R	5'-GTC ATA TTC ATT TAA CAC ACT ATT ATT ACC	-	2186[b]	sequencing of PAI
PAI-17R	5'-CGA TTT CCT TAG TAT AAT AAA CAA TC	-	1665[b]	sequencing of PAI; PCR
PAI-18R	5'-TTT GCA ATG AAT TAT AGA GTC G	-	1170[b]	sequencing of PAI
PAI-19R	5'-AAT CTA TAC ACG AAT AAG AAT ATT ATC C	-	438[b]	sequencing of PAI
PAI-20R	5'-GAG AAA ACA TTG ATA ATA GTC CAG	-	10145[b]	sequencing of PAI
PAI-21R	5'-ATG TAT TCC ATT TTT TGA TAG TAT TTC	-	9547[b]	sequencing of PAI
PAI-2F-Biotin	5'-GTA TTA GCG GTG TTC AAA ATG	+	49[b]	Southern hybridisation; detection *orf1*
esp $_{fm}$1R-Biotin	5'-GTA ATT AGC ATA CCA AGG CG	-	4068[b]	Southern hybridisation; detection *esp*
PAI-10F-Biotin	5'-AAA ATA GTC ACT ACA AGT GGT ACC C	+	9599[b]	Southern hybridisation; detection *orf4*
PAI-11F-Biotin	5'-TTG CAT CAG CAG TTA TAT TAA TG	+	10401[b]	Southern hybridisation; detection *orf5;* PCR
PAI-8F-Biotin	5'-ACC GAA AAA TAA TAC AAG TGG	+	12760[b]	Southern hybridisation; detection *orf6*
PAI-4F-Biotin	5'-CTA GTA TGA CTA TGG CTA CAA ATG C	+	13753[b]	Southern hybridisation; detection *orf7*

[a] Nucleotide reference positions relative to the *E. faecalis esp* sequence (GenBank, EMBL database accession no. AF034779)

[b] Nucleotide reference positions relative to the *E. faecium* PAI sequence deposited in the GenBank/EMBL database under accession no. AY322150

blood agar plates at 37°C for further use.

The following isolates were used to determine the presence of the putative PAI in *E. faecium*: isolates from hospital outbreaks Australia-1, NL-1-1, NL-2-1, NL-2-3, NL-3-1, UK-1, US-1, US-2-1, US-2-2, US-2-3, US-2-4, US-2-5, US-2-6, and US-2-7 (4, 5, 11, 28, 35, 58, 64, 65); 68 clinical isolates (44 from blood, 9 from pus, 7 from urine, 5 from peritoneal fluid, 1 from bile, 1 from lungs, and 1 from skin) (4, 11, 16, 48, 64, 67) from the SENTRY Antimicrobial Surveillance Program, originating from hospitals in 15 different countries (Australia, Austria, Belgium, France, Germany, Israel, Italy, The Netherlands, Poland, Portugal, Spain, Switzerland, Turkey, the United Kingdom, and the United States); 6 hospital surveillance isolates (feces isolates with no link to a hospital outbreak) from three different countries (France, The Netherlands, and the United Kingdom) (28, 48, 58, 64, 66); 3 community surveillance isolates from The Netherlands (feces isolates with no hospital link) (16, 62, 67); and 10 animal feces isolates from The Netherlands (2 each from cats, dogs, calves, swine, and poultry) (61-63, 66).

PCR and sequencing of the *E. faecium esp* gene

The nonrepeat regions of the *E. faecium esp* gene were amplified and sequenced by using a combination of 17 primers based on the published *E. faecalis esp* sequence (GenBank/EMBL accession no. AF034779) (51) and 4 primers based on the *E. faecium* sequence determined in this study (Table 1). Chromosomal DNA was purified as described elsewhere (66, 67). PCR conditions for all amplification reactions were as follows: initial denaturation at 95°C for 15 min, followed by 35 cycles of 30 s at 94°C, 30 s at 52°C, and 30 s at 72°C, and a final 5-min extension at 72°C. Reactions were performed in 25-μl volumes with HotStar *Taq* polymerase and HotStar Master Mix buffers (Qiagen Inc., Valencia, Calif.). PCR products were purified with a PCR purification kit (Qiagen Inc.) and sequenced by using the BigDye Terminator reaction kit and an ABI PRISM 3700 DNA analyzer (both from Applied Biosystems, Foster City, Calif.).

For sequencing of the region encompassing the A and C repeats, a slightly different approach was followed. First the A- and C-repeat regions were amplified with the primer combinations *esp*$_{fs}$6F-*esp*$_{fs}$4R and *esp*$_{fs}$9F-*esp*$_{fs}$2R, respectively, and were subsequently cloned into pCR2.1-TOPO by using the TOPO TA cloning kit (Invitrogen Life Technologies, Carlsbad, Calif.) in accordance with the manufacturer's instructions. This resulted in pJT1 and pJT2, harboring the A- and C-repeat regions, respectively. To generate subclones suitable for sequencing, overlapping deletions were constructed with the Erase-a-base system (Promega Corporation, Madison, Wis.). Subclones were sequenced by using the M13 reverse primer, the BigDye Terminator reaction kit, and an ABI PRISM 3700 DNA analyzer (all from Applied Biosystems).

The 5' end of the *esp* gene was amplified by a combination of primer *esp*$_{fs}$10R and an 18-mer primer consisting of thymidines only. This PCR fragment was cloned into pCR2.1-TOPO by using the TOPO TA cloning kit (Invitrogen Life Technologies) in accordance with the manufacturer's instructions, and the resulting plasmid, designated pJT3, was sequenced using primers *esp*$_{fs}$6R, *esp*$_{fs}$10R, and *esp*$_{fm}$1F. Clone pJT2 was also used to determine the nucleotide sequence of the 3' end of the *esp* gene, since primer *esp*$_{fs}$2R is located just downstream of the *esp* gene.

Determination of variation in the *esp* A and C repeats

Two different primer combinations were used to assess repeat number variation by PCR. Primer sets *esp*$_{fs}$7F-*esp*$_{fm}$5R and *esp*$_{fm}$5F-*esp*$_{fs}$3R (Table 1) were used to amplify across the A- and C-repeat regions of the *esp* gene, respectively, in a set of 36 *E. faecium* isolates. Amplification conditions were identical to those described above. Subsequently, the amplicons were subjected to agarose gel electrophoresis (1%) in order to determine their sizes. From the sizes of the amplicons the numbers of repeats were deduced. Amplicon size differences corresponded to multiples of either 252 bp (A repeats) or 246 bp (C repeats).

Cloning and sequencing of the putative PAI

The DNA region adjacent to *esp* was cloned by an inverse-PCR strategy. Approximately 10 μg of chromosomal DNA was digested with *Eco*RI or *Bcl*I, and the resulting fragments were self-ligated. Ligated DNA was amplified with primer *esp*$_{fm}$4R, located in the 5' end of the *esp* gene, and primer *nox*1F, located just downstream of the *esp* gene, by using the Expand Long Template PCR system (Roche Diagnostics Nederland B.V., Almere, The Netherlands). Six-kilobase *Bcl*I and 7.9-kb *Eco*RI inverse-PCR products were cloned into pCR2.1-TOPO by using the TOPO TA cloning kit (Invitrogen Life Technologies) in accordance with the manufacturer's instructions, producing plasmids pJT4 (*Eco*RI digest) and pJT5 (*Bcl*I digest). Overlapping deletions of pJT4 and pJT5 were constructed with the Erase-a-base system (Promega) to generate subclones suitable for sequencing. One strand of the pJT4 and pJT5 subclones was sequenced with the M13 forward primer in combination with the BigDye Terminator reaction kit by using an ABI PRISM 3700 DNA analyzer (all from Applied Biosystems). Gaps in the DNA sequence of the first strand and sequence information of the second strand were obtained by direct sequencing of PCR products with primers based on the emerging nucleotide sequence of the first strand. Primers that were used for PCR and sequencing of this DNA region are listed in Table 1. PCR conditions were the same as those described above.

Detection of the putative PAI in *E. faecium* isolates

Southern hybridization was used to determine the presence of six open reading frames (ORFs) contained in the putative PAI in a set of 105 *E. faecium* isolates. For this purpose, chromosomal DNA preparations were digested with *Hae*III, separated by agarose gel electrophoresis (0.7% agarose gels), transferred onto a Hybond N^+ nylon membrane (Nycomed Amersham plc, Little Chalfont, Buckingham, United Kingdom), and subsequently hybridized to six biotin-labeled oligonucleotide probes specific for the six ORFs according to the protocol developed by Schouls and coworkers (47a). The oligonucleotides used as probes for hybridization are listed in Table 1.

Nucleotide sequence accession numbers

The DNA sequences reported in this article have been deposited in the GenBank/EMBL/DDBJ nucleotide sequence databases under accession no. AY322150 (*E. faecium* E300 putative PAI), AY322497 (*E. faecium* E155 hypothetical phage gene), AY322498 (*E. faecium* E734 permease gene), AY322499 (*E. faecium* E734 *esp* 5' end), AY322501 (*E. faecium* E734 *esp* 3' end), AY322500 (*E. faecium* E470 *esp* 5' end), and AY322502 (*E. faecium* E470 *esp* 3' end).

Results

Sequence analysis of the *E. faecium esp* gene

In an attempt to obtain the DNA sequence of the *esp* gene in *E. faecium*, a DNA region of strain E300, which was recovered during a hospital outbreak (11, 65), was amplified by PCR using primers based on the *E. faecalis esp* sequences (Table 1), followed either by direct sequencing of the PCR products or by making overlapping deletions of a cloned amplicon followed by sequencing of the deletion mutants. Sequence analysis revealed one ORF of 5,703 nucleotides that is predicted to encode a polypeptide of 1,900 amino acid residues with a calculated molecular mass of 205 kDa.The deduced amino acid sequence of the *E. faecium* Esp protein revealed a high degree of similarity to, but appeared not to be identical with, the recently described Esp of *E. faecium* strain P61 and the *E. faecalis* Esp protein (51). The *E. faecium* E300 Esp is predicted to be synthesized as a precursor with a 49-amino-acid signal peptide that precedes an N-terminal region of 706 amino acids, a central repeat region, and a C-terminal domain (Figure 1). The N-terminal domain has 99 and 91% amino acid sequence identities with *E. faecium* P61 Esp and *E. faecalis* Esp, respectively. Remarkably, the first 23 amino acid residues of the processed protein of E300 are highly different from those of *E. faecalis* Esp. The central repeat region of the variant E300 Esp protein contains five A repeats of 84 amino acids, followed by one B1 repeat (79 amino acids), five C

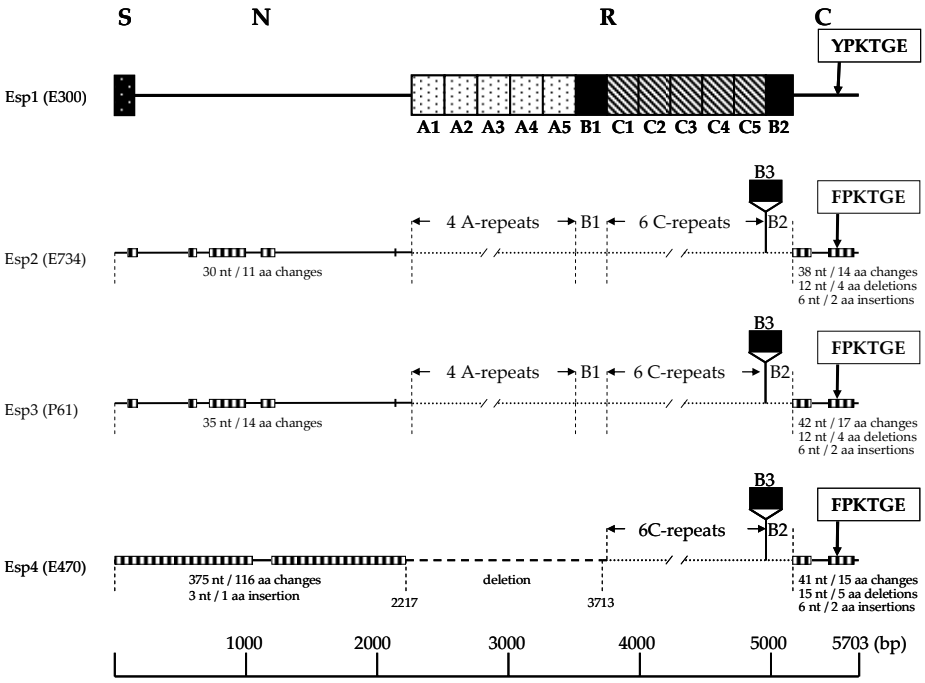

Figure 1. Schematic representation of the inferred *E. faecium* Esp protein and comparison of four *E. faecium* Esp variants. Esp1, deduced sequence of the Esp protein of strain E300, comprising the signal sequence (S) (solid box with white dots), N-terminal region (N), repeat region (R), and C-terminal region (C). The A-, B-, and C-repeat units are indicated (dotted, solid, and crosshatched boxes, respectively). YPKTGE and FPKTGE, anchor motifs in the C-terminal region. Solid lines in Esp2 (accession no. AF444000, AY322499, and AY322501), Esp3 (accession no. AJ487981), and Esp4 (accession no. AY322500 and AY322502) represent regions for which the DNA and amino acid sequences were compared to those of Esp1 (accession no. AF443999 and AY322150), while dotted lines represent regions that were not compared. Striped boxes in Esp2, -3, and -4 indicate locations of nucleotide (nt) and amino acid (aa) changes, with numbers of nucleotide and amino acid changes, insertions, and deletions relative to Esp1 indicated below. The dashed line in Esp4 represents the deletion in the *esp* gene of E470. The start and end points of this deletion, positions 2217 and 3713, respectively, relative to the E300 *esp* sequence, are indicated.

repeats (82 amino acids), and one B2 repeat (68 amino acids) (Figure 1). The beginning and end of the B and C repeats were chosen slightly differently from those published by Shankar et al. (51) and Eaton and Gasson (13), so that only complete instead of truncated copies of C repeats are present in the central part of the *esp* gene (Figure 2). The repeats in *E. faecium* E300 are highly similar to those of *E. faecium* P61 Esp and *E. faecalis* Esp, with amino acid identities of 98 to 99% for the A repeats, 96 to 98% for the B1 repeat, 97 to 98% for the C repeats, and 87 to

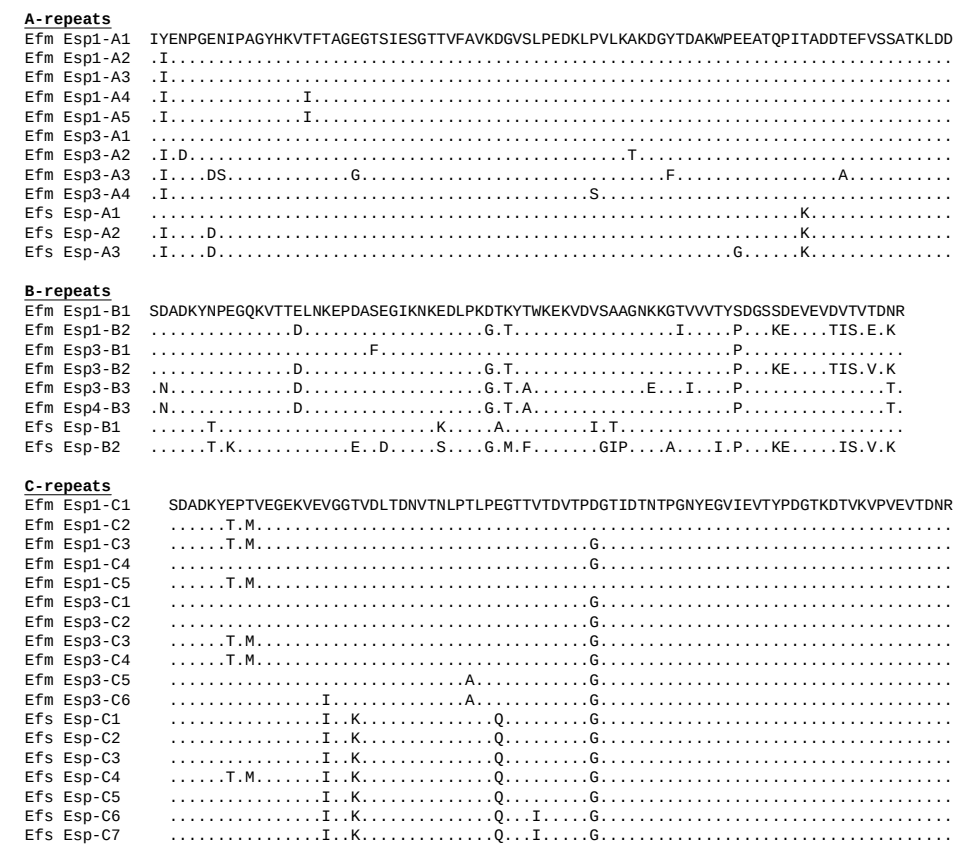

Figure 2. Comparison of the primary structures of the A, B, and C repeats of *E. faecium* Esp1 (E300), *E. faecium* Esp3 (P61) (13), and *E. faecalis* Esp (51) and the B3 repeat of *E. faecium* Esp4 (E470). Dots indicate identical amino acid residues. Only those amino acid residues of Esp3, Esp4, and *E. faecalis* Esp that differ from the repeats of *E. faecium* Esp1 are represented by letters. Efs, *E. faecalis*; Efm, *E. faecium*.

99% for the B2 repeat. *E. faecium* E300 Esp lacked the third B repeat (B3) that was reported for the P61 Esp (Figure 1 and 2).

The C-terminal domain of 167 amino acid residues contains a membrane-spanning hydrophobic region, the YPKTGE cell wall anchor motif, and a charged tail presumably extending into the cytoplasm, ending with a glutamic acid. This domain is also highly similar to those of *E. faecium* P61 Esp and *E. faecalis* Esp, with 87 and 84% amino acid identities, respectively. The overall similarities of *E. faecium* E300 Esp with the *E. faecium* P61 and *E. faecalis* Esp proteins, disregarding the number of repeats, are 96 and 92%, respectively.

Sequence heterogeneity in the *E. faecium esp* gene

In a previous study, sequence heterogeneity was identified in an internal fragment of the *E. faecium esp* gene (65). To determine sequence heterogeneity in *E. faecium esp* genes in more detail, the DNA regions encoding the N- and C-terminal domains of two additional *esp* genes from two outbreak-related vancomycin-resistant *E. faecium* isolates (E734 and E470) were amplified and sequenced, and the DNA sequences were compared to the corresponding E300 and P61 *esp* sequences. These comparisons revealed considerable polymorphism in the DNA regions encoding the N- and C-terminal domains, resulting in four different copies of the *E. faecium esp* gene, designated *esp1* to *esp4*, tentatively encoding four different Esp proteins, Esp1 to Esp4; *esp1* is the sequenced *esp* gene of strain E300, and *esp3* is the sequenced *esp* gene of *E. faecium* P61 (13) (Figure 1). The *esp2* gene was found in strain E734 from outbreak NL-1 and harbored 70 nucleotide differences from *esp1*, resulting in 26 amino acid changes. Furthermore, a 4-amino-acid deletion and a 2-amino-acid insertion, relative to the Esp1 protein, were found in the C-terminal domain of Esp2, as well as a third copy of the B3

Table 2. Variations in A-, B-, and C-repeats in *esp* analyzed in 36 *E. faecium* isolates

Source	No. of strains analyzed	Country[a]	No. of repeats			*esp* repeat profile
			A	C	B3	
Clinical isolate	1	PO	6	5	1	1
Clinical isolate	1	AU	6	5	1	1
Clinical isolate	1	GR	4	6	1	2
Clinical isolate	1	GR	5	6	1	3
Clinical isolate	1	GB	6	6	1	4
Clinical isolate	1	IT	4	6	1	2
Clinical isolate	1	FR	4	6	1	2
Clinical isolate	1	GB	5	6	1	3
Clinical isolate	1	AU	5	4	1	5
Hospital outbreak NL-1-1	10	NL	4	6	1	2
Hospital outbreak NL-2-1	2	NL	4	6	1	2
Hospital outbreak NL-3	1	NL	0	6	1	6
Hospital outbreak UK-1	4	UK	6	6	1	4
Hospital outbreak US-1	2	US	3	5	0	7
Hospital outbreak US-2-1	1	US	0	6	1	6
Hospital outbreak US-2-2	1	US	0	6	1	6
Hospital outbreak US-2-3	1	US	0	6	1	6
Hospital outbreak US-2-4	1	US	3	6	0	8
Hospital outbreak US-2-5	1	US	3	6	1	9
Hospital outbreak US-2-6	1	US	3	6	1	9
Hospital outbreak US-2-7	1	US	3	6	1	9
Hospital survey	1	GB	6	7	1	10

[a] PO, Poland; AU, Austria; GR, Greece; IT, Italy; FR, France; NL, The Netherlands; UK, United Kingdom; US, United States.

repeat. Also, the FPKTGE cell wall anchor motif in the C-terminal domain of Esp2 was different from that in Esp1 but identical to the anchor motif found in the P61 Esp3 protein (13). In general, the *esp*2 gene closely resembled the P61 *esp3* gene:the sequenced regions of *esp2* differed by only 11 nucleotides from *esp3*. The *esp4* gene, found in strain E470 from outbreak NL-3, contained 416 nucleotide differences in the regions encoding the N- and C-terminal domains relative to *esp1*, resulting in 131 amino acid changes, with most of the differences found in the region encoding the N-terminal domain. In addition to nucleotide changes, the *esp4* gene contained a large deletion in the region encoding the N-terminal domain, which also included the entire A-repeat region.

In addition to the observed nucleotide differences, the repeat regions of Esp appeared to be highly polymorphous, with variations in the numbers of A, B, and C repeats. This is not unexpected, since polymorphisms in these regions have been reported before in *E. faecalis* and *E. faecium* (13, 51). Thirty-six *E. faecium* isolates were analyzed for the numbers of A and C repeats. The number of A repeats varied from 0 to 6, while the number of C-repeats varied from 4 to 7, resulting in 10 different *esp* repeat profiles (Table 2). All strains originating from a single outbreak had identical repeat regions. Ten of the isolates, from outbreak NL-1-1, were collected during a 2-year period between April 2000 and April 2003, and they were all indistinguishable with respect to the number of repeats. In addition, the Esp repeat profile of these isolates was identical to that of the two isolates from outbreak NL-2-1, which previously had been shown to be epidemiologically linked to outbreak NL-1-1 (35). This Esp repeat profile of the Dutch outbreak strains was also found in epidemiologically unrelated clinical isolates from Greece, Italy, and France. This finding suggests that Esp repeat profiles are relatively stable, at least among strains associated with a single outbreak.

A cluster of genes adjacent to the *E. faecium esp* gene

Recently, it was reported that the *esp* gene of *E. faecalis* was part of a large (150-kb) PAI (49). To examine whether the *esp* gene in *E. faecium* was also present on a PAI, an inverse-PCR strategy was used on *Bcl*I- and *Eco*RI-digested chromosomal DNA to obtain sequence information for a 14-kb DNA fragment. Sequencing of this DNA fragment revealed seven ORFs including the *esp* gene (Figure 3). A search for homology using the GenBank/EMBL database showed that the predicted amino acid sequence of ORF1 (41 amino acids) had 42% similarity to the N-terminal part of the Uve2 protein encoded by the *vanE* gene cluster (Table 3). From this similarity it was also clear that only a part of this putative gene was cloned and sequenced. The *uve2* gene contained in the *vanE* gene cluster is 26% identical to the sigma factor SpoIIG of *Bacillus subtilis* (6). The *orf2* gene is

Table 3. GC contents of the 7 ORFs contained in the putative *E. faecium* PAI and maximum predicted amino acid similarities

Locus	GC content (%)	Homologue	Maximum amino acid similarity of predicted protein to homologue (%)
orf1	32.5	*E. faecalis* Uve2	42
orf2	27.9	*E. faecalis* AraC	61
orf3	38.1	*E. faecalis* Esp	92
orf4	28.9	*E. faecalis* NADH Oxidase	35
orf5	35.6	*S. pyogenes* muramidase	27
orf6	32.8	*L. monocytogenes* bacteriophage protein	33
orf7	43.6	*L. lactis* MDR protein	45

predicted to encode a 401-amino-acid protein. This putative gene exhibited similarity with the *araC* gene found in the *E. faecalis* PAI (Table 3).

The *esp1* gene, the third ORF in this gene cluster, which is described in detail above, is present downstream of the *araC* gene (Figure 3). The *orf4* gene is predicted to encode a 447-amino-acid protein. This putative gene is located just downstream of *esp* and displays the highest similarity with the *nox* gene of *E. faecalis*, encoding an NADH oxidase. Although the overall similarities with homologous proteins in *Streptococcus pneumoniae*, *E. faecalis*, and *S. pyogenes* are relatively low (29.3, 34.8, and 33.8%, respectively), the three sequence motifs representing the flavin adenine dinucleotide (FAD) binding region, the NADH contact region, and a cysteine residue essential for redox activity are conserved in the putative *E. faecium* NADH oxidase present in this gene cluster (2, 19, 45) (Figure 3C).

The *orf5* product is predicted to be synthesized as a 483-amino-acid precursor with an amino-terminal signal sequence of 27 amino acid residues and shows similarity with peptidoglycan hydrolases, *N*-acetylmuramidases, and autolysins of *E. faecalis*, *Enterococcus hirae*, *Lactococcus lactis*, and *S. pyogenes* (Table 3; Figure 3). Alignment of the *E. faecium* putative muramidase polypeptide with the muramidase-2 gene product of *E. hirae* and the *E. faecalis* autolysin reveals that similarity is restricted to the N-terminal enzymatically active domain and that the *E. faecium* putative muramidase protein lacks the C-terminal peptidoglycan anchor domain (29). These findings make it unclear whether this gene encodes a functional muramidase or autolysin. In addition, the putative muramidase also contains the S144SKK, S178GN, D258/E282, and K354TG motifs found in serine ß-lactamases and penicillin-binding proteins (18). This could mean that this *E.*

faecium protein also displays penicillin binding properties comparable to those of the muramidase-2 protein of *E. hirae* (10, 29).

Downstream of the putative muramidase gene are two small ORFs displaying similarity with phage-associated hypothetical proteins of *Lactobacillus* spp., *Listeria monocytogenes*, *S. pyogenes*, and *Pseudomonas aeruginosa*. Detailed examination of the sequence at positions 12830 to 12832 suggests the presence of a frameshift in isolate E300. Sequencing of this region in the epidemic *E. faecium* isolate E155 (US-2-6) (65) showed the presence of an extra nucleotide ("A") at position 12832 and confirmed that in this isolate the two ORFs in fact belong to one single ORF, *orf6*, which is predicted to encode a 256-amino-acid protein with a calculated molecular weight of 29,369. The deduced amino acid sequence of ORF6 revealed the highest similarity, 33%, with an unknown bacteriophage protein of *L.*

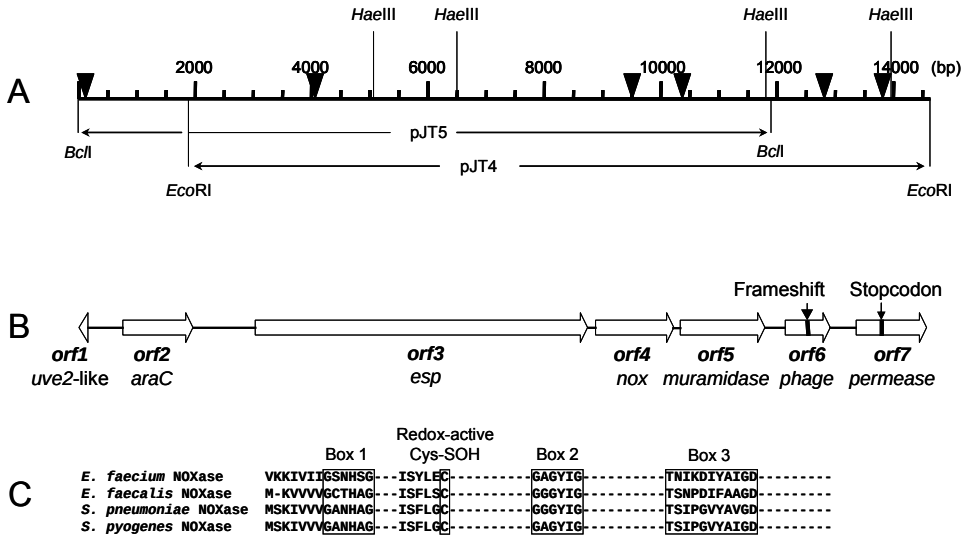

Figure 3. Schematic representation of the putative PAI in *E. faecium* and alignment of NADH oxidase regions. (A) Genetic map of the PAI. Numbers correspond to base pair positions relative to accession no. AY322150. Only restriction enzyme recognition sites relevant for this study are shown. The locations of the two clones that were constructed to derive the entire sequence are indicated. Arrowheads indicate the positions of the oligonucleotide probes used in the Southern hybridization. (B) Physical map of the PAI. Large open arrows with proposed names below indicate sizes, locations, and orientations of predicted ORFs. The positions of the frameshift and stop codon in the PAI of strain E300 are indicated. (C) Comparison of sequence fingerprints of the FAD binding region (boxes 1 and 3), the NADH contact region (box 2), and the cysteine-sulfenic acid redox center of the NADH oxidases (NOXase) of *E. faecium* (this study) with three previously identified homologues: *E. faecalis* (GenBank accession no. X68847) (45), *S. pneumoniae* (GenBank accession no. AF014458) (2), and *S. pyogenes* (GenBank accession no. AF101442) (19).*monocytogenes* strain EGD-e (Table 3; Figure 3) (21).

The last ORF, *orf7*, exhibited amino acid sequence identity with multidrug resistance permeases of *Clostridium perfringens* and *L. lactis*, suggesting that the *orf7* gene may encode a multidrug resistance efflux pump (Table 3; Figure 3). In E300, *orf7* was interrupted by a stop codon at position 13719. Again, repeated sequencing of this region in *E. faecium* isolate E734, which belonged to hospital outbreak NL-1-1 (65), demonstrated that in this isolate the sequence TTA, encoding a leucine, was present instead of the TAA stop codon observed in strain E300. The fact that no stop codon was found in the uninterrupted *orf7* gene suggests that only a part of this gene is present in the cloned and sequenced copy of the *E. faecium esp* gene cluster.

The *E. faecium esp* gene cluster is part of a putative PAI

To investigate whether there was a physical link between *esp* and the other ORFs in this gene cluster, *E. faecium* isolates carrying *esp* and *esp*-deficient strains were analyzed for the presence of the other six ORFs by Southern hybridization. Chromosomal DNAs of 105 *E. faecium* isolates were digested with *Hae*III and hybridized to six oligonucleotide probes derived from internal parts of, and specific for, *orf1* and *orf3* to *orf7* (Figure 3). The selection included 50 vancomycin-susceptible and 55 vancomycin-resistant (*vanA*-positive) isolates (Table 4). All 26 isolates that were *esp* positive also reacted with all six oligonucleotide probes, while all 79 *esp*-negative isolates failed to react with any of the six oligonucleotides. This shows that in this set of isolates, the entire *esp* gene cluster was either present or absent. The 26 isolates carrying this gene cluster included epidemic and clinical isolates, while this cluster was absent in all surveillance and animal isolates. Furthermore, the hybridization results showed that *orf1* and *orf3* were located on identical-sized DNA fragments, as were *orf4* and *orf5*, and *orf6* and *orf7* (data not shown). To further examine whether ORFs 1 to 7 are located in proximity to each other, PCRs were performed with forward and reverse primers specific for different ORFs. PCRs with the primer combinations PAI2F (*orf1*)-PAI17R (*orf2*), esp_{fs}9F (*orf3*)-*nox*2R (*orf4*), PAI11F (*orf5*)-PAI7R (*orf6*), and PAI11F (*orf5*)-PAI3R (*orf7*) (Table 1) demonstrated that ORFs 1 and 2, ORFs 3 and 4, ORFs 5 and 6, and ORFs 6 and 7 are located adjacent to each other (data not shown).

The observation that all six of the putative genes are either present or absent and are physically linked on the genome strongly suggests that this cluster of genes is part of a PAI. This is also supported by the fact that several of the putative genes in this gene cluster have a deviant GC content, ranging from 27.9 to 43.6%, compared to the average GC content of 37.8% in *E. faecium* (Table 3; http://www.jgi.doe.gov/JGI_microbial/html/enterococcus/enterococcus_homepage.html).

Discussion

In this study we have identified a cluster of six genes in *E. faecium* that are potentially involved in regulation, virulence, and antibiotic resistance and are linked to the *esp* virulence gene. We have designated this gene cluster a putative PAI because of (i) the Southern blot analysis of *esp* positive and -negative *E. faecium* isolates, which demonstrated the presence or absence of the entire gene cluster and a physical link among the seven putative genes in this cluster; (ii) the deviant GC content of this gene cluster (34.4%) compared to the GC content of the

Table 4. Presence of a putative PAI among vancomycin-susceptible and -resistant *E. faecium* isolates from different sources

Strain origin	No. of isolates			
	PAI-		PAI+	
	VSEF[a]	VREF[b]	VSEF	VREF
Hospital outbreak isolates:				
AU-1				1
NL-1-1				1
NL-2-1				2
NL-2-3		1		
NL-3-1				1
UK-1				2
US-1				3
US-2-1				1
US-2-2				1
US-2-3				1
US-2-4				1
US-2-5				1
US-2-6				1
US-2-7				1
Clinical isolates	42	17	8	1
Hospital surveillance isolates		6		
Community surveillance isolates		3		
Pet isolates (dogs, cats)		4		
Swine isolates		2		
Poultry isolates		2		
Calf isolates		2		
Total	42	37	8	18

[a] Vancomycin-susceptibel *E. faecium*

[b] Vancomcyin-resistant *E. faecium*

E. faecium strain DO chromosome (37.8%); (iii) the presence of putative virulence genes; and (iv) the fact that this island is absent in all human surveillance and animal isolates but present in epidemic and clinical isolates. The large variation in GC content of the seven ORFs present in the putative PAI, ranging from 27.9 up to 43.6%, suggests that this island was generated not as a result of one single event but as a result of a complex evolution involving multiple steps in different bacterial ancestors, and that it was finally acquired by *E. faecium* through horizontal gene transfer.

The presence of the putative PAI seems to be associated with epidemicity, since 13 of the 14 clones analyzed from different hospital outbreaks contained this PAI. This finding is in line with previous findings that suggested the existence of an epidemic *E. faecium* subpopulation with specific genetic characteristics (65). The fact that such a subpopulation is characterized not only by the presence of *esp* but also by the acquisition of a large genomic island may improve rapid detection of potential epidemic *E. faecium* strains, thus facilitating rapid implementation of infection control strategies. Furthermore, proteins encoded by the putative PAI may be potential targets for specific therapies, for example, to eradicate or prevent gastrointestinal colonization by potentially epidemic *E. faecium.*

A homologue of *E. faecium esp* contained in this putative PAI was first described in *E. faecalis*, where it was found in a high proportion of clinical strains (51). Recently it was shown that the *E. faecalis esp* gene is part of a large (150-kb) PAI (49). The *E. faecalis esp* gene encodes a surface-exposed protein and is thought to be involved in colonization of the urinary tract (50) and biofilm formation (59). In *E. faecium*, the *esp* gene was initially found in vancomycin-resistant outbreak-related isolates (65); later, it was also found in vancomycin-susceptible clinical isolates (3, 12, 13, 68). Recently, the *E. faecium* gene from a clinical isolate, P61, was cloned and sequenced by Eaton and Gasson (13). It displayed 89% similarity with the *E. faecalis esp* gene. It also exhibited global structural similarity to the *S. agalactiae* Rib and alpha C proteins, the R28 protein of *S. pyogenes*, and the biofilm-associated protein (Bap) of *S. aureus*, all of which are known virulence factors conferring protective immunity (8, 33, 37, 54, 55). All these proteins contain a repeat region in which amino acid similarities are most prominent (13, 51). The *E. faecium esp* genes analyzed in this study were highly similar but not identical to the P61 *esp* gene. In addition to variations in the numbers of A, B, and C repeats, extensive polymorphism was found in the N- and C-terminal nonrepeat regions. This may suggest that the *esp* gene was not acquired recently by *E. faecium* or that the *esp* gene is a relatively "ancient" gene acquired by *E. faecium* during multiple occasions. In addition, heterogeneity, especially in the surface-exposed N-terminal region, may correspond to different functions or specificities of different Esp variants. Differences in repeat numbers in *esp*, both in *E. faecalis* and in *E.*

faecium, have been reported previously (13, 51). It is questionable whether this heterogeneity in repeat numbers can be used as an epidemiological tool. Comparison of the *esp* repeat profiles of epidemiologically linked and unrelated strains suggests that *esp* repeat profiling may be used to study local outbreaks but probably does not discriminate sufficiently to serve as a major tool for global epidemiology unless it is used in combination with genotyping schemes such as multilocus sequence typing or pulsed-field gel electrophoresis.

The presence of the *esp* gene in isolates from epidemiologically distinct sources seems to differ between *E. faecalis* and *E. faecium*. While the presence of the *esp* gene in *E. faecium* is confined to clinical and epidemic isolates, in *E. faecalis* the *esp* gene is also found in isolates from farm animals and food (12, 17, 24). This could be related to differences in the frequency of horizontal transmission of the *esp* gene in *E. faecalis* and *E. faecium*.

In addition to *esp*, two other putative virulence genes were found on this genetic island: the *nox* and muramidase genes, encoding a NADH oxidase and muramidase or autolysin, respectively. NADH oxidases are enzymes that can catalyze the four-electron reduction of O_2 to H_2O and are considered to perform normal household functions. In *E. faecalis*, NADH oxidase is involved in glycolytic metabolism (47). However, similar enzymes in *S. pyogenes*, *Streptococcus mutans*, and *S. pneumoniae* are considered virulence factors involved in adaptive responses to O_2, enabling these bacteria to grow in O_2-rich environments (2, 19, 25, 69). Furthermore, the NADH oxidase of *S. pneumoniae* is also involved in natural competence for genetic exchange (2, 14). It is not yet known whether the NADH oxidase found on the *E. faecium* putative PAI is involved in virulence. One can speculate that *E. faecium* isolates harboring this enzyme are better equipped to leave the anaerobic conditions in the gut and grow in more oxygen rich niches such as the urinary tract or the bloodstream.

The muramidase gene is predicted to encode an enzyme with important physiological functions during cell growth and division (52, 53, 56). Most of these enzymes have a domain structure (42). The *E. faecium* muramidase encoded by the putative PAI displayed similarity only with the N-terminal enzymatically active domain of the *E. hirae* muramidase-2 and seems to lack the C-terminal peptidoglycan binding domain. It was shown previously that the muramidase-2 enzyme of *E. hirae* covalently binds penicillin (10). It is not known whether the *E. faecium* muramidase described here is able to bind ß-lactam antibiotics, but the characteristic motifs present in serine ß-lactamases and penicillin-binding proteins are also conserved in this protein. In addition to basic cell functions, some bacterial peptidoglycan hydrolases, muramidase or autolysin, have been implicated in virulence by contributing to primary adhesion, biofilm formation, or other, yet unknown processes (1, 22, 27, 31, 36, 39, 46). Some other murein

hydrolases, such as the lysostaphin of *Staphylococcus simulans*, may also act as bacteriocins (70). The production of bacteriocins may provide a competitive advantage in specific niches, thus promoting intestinal colonization. Furthermore, a peptidoglycan hydrolase gene of *Neisseria gonorrhoeae*, *atlA*, was also found on a PAI (9), and it was demonstrated that this *atlA* gene was required for DNA secretion during growth. This suggests that peptidoglycan hydrolases may also play a role in DNA transfer events. Further characterization of the peptidoglycan hydrolase encoded by the muramidase-like gene on the putative PAI is needed to establish a potential role in penicillin binding, pathogenesis, or intestinal colonization.

The first two ORFs of this putative PAI encode putative transcriptional regulators. *orf1*, which was cloned and sequenced only partially, may encode a sigma-like factor, while *orf2* most likely encodes a protein that belongs to the AraC family of global regulators. Both AraC and alternate sigma factors are often found on PAIs (reviewed by Hacker and Kaper [23] and Egan [15]). Interestingly, an *araC*-like gene was also found on the recently described *E. faecalis* PAI, downstream of *esp*, while the *E. faecium araC* is located upstream of *esp* (49). Transcriptional regulators contained on PAIs may regulate virulence genes located on the same island or genes located outside the PAI. At this moment the role of these regulators in *E. faecium* is the subject of ongoing research.

The last two ORFs were disrupted in isolate E300 but were found intact in other isolates. They may encode a hypothetical bacteriophage protein and a multidrug resistance efflux pump. Bacteriophages have been implicated in the mobilization of PAIs, and several PAIs contain sequences with homology to bacteriophage integrase genes (reviewed by Hacker and Kaper [23]). The exact function of this putative phage protein remains to be elucidated. Sequence analysis and alignment of the last ORF suggested that *orf7* may encode a putative multidrug resistance efflux pump that was only partially present on the cloned and sequenced copy of the putative PAI. Although virulence and antibiotic resistance may often be linked (34), antibiotic resistance genes are rarely found on PAIs. Recently, a PAI carrying a resistance locus conferring resistance to streptomycin, ampicillin, chloramphenicol, and tetracycline was found in *Shigella flexneri* (32, 60).

Comparison of the putative *E. faecium* PAI and the recently published *E. faecalis* PAI revealed that these two enterococcal PAIs are different, although they share at least two genes: *araC* and *esp* (49). It is intriguing that these two related enterococcal species, which are often found in the same niche, carry different PAIs. On the other hand, the epidemiology of the two species seems to be different. While *E. faecalis* is more frequently encountered among clinical isolates, *E. faecium*, mainly ampicillin- and vancomycin-resistant isolates, is more often

associated with epidemic spread in hospitals (26, 40, 43). It is possible that differences in PAI sequences between *E. faecium* and *E. faecalis*, in addition to differences in antibiotic susceptibility, may account for these epidemiological differences.

Since PAIs may provide a rapid and flexible means of evolution of virulence by generating new pathogenic variants, it is not unlikely that the acquisition of a PAI by *E. faecium* has played an important role in the rapid emergence of *E. faecium* as a nosocomial pathogen.

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Chapter 6

Global Spread of Vancomycin-resistant *Enterococcus faecium* from Distinct Nosocomial Genetic Complex

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Abstract

Vancomycin-resistant enterococci (VRE) have caused hospital outbreaks worldwide, and the vancomycin-resistance gene (*vanA*) has crossed genus boundaries to methicillin-resistant *Staphylococcus aureus*. Spread of VRE, therefore, represents an immediate threat for patient care and creates a reservoir of mobile resistance genes for other, more virulent pathogens. Evolutionary genetics, population structure, and geographic distribution of 411 VRE and vancomycin-susceptible *Enterococcus faecium* isolates, recovered from human and nonhuman sources and community and hospital reservoirs in 5 continents, identified a genetic lineage of *E. faecium* (complex-17) that has spread globally. This lineage is characterized by 1) ampicillin resistance, 2) a pathogenicity island, and 3) an association with hospital outbreaks. Complex-17 is an example of cumulative evolutionary processes that improved the relative fitness of bacteria in hospital environments. Preventing further spread of this epidemic *E. faecium* subpopulation is critical, and efforts should focus on the early disclosure of ampicillin-resistant complex-17 strains.

Introduction

The emergence of vancomycin-resistant enterococci (VRE) followed a worst-case scenario for nosocomial pathogens: the first VRE isolates that harbored the *vanA* transposon were identified in 1987 in Europe (1,2), and within 10 years VRE represented >25% of enterococci associated with bloodstream infections in hospitalized patients in the United States (3).

Enterococci are normal inhabitants of the gastrointestinal tract of humans and animals. Two species cause most enterococcal infections, *Enterococcus faecalis* and *E. faecium*. The relative importance of *E. faecium* as a pathogen has increased with the occurrence of high-level resistance to multiple antimicrobial drugs, such as ampicillin and vancomycin (4). The rapid increase of vancomycin resistance compromises physicians' ability to treat infections caused by many of these strains because often no other antimicrobial drugs are available. The epidemiology of VRE infection differs between Europe and the United States. In Europe, VRE are frequently isolated from farm animals, which have been associated with the abundant use of avoparcin as a growth promoter in the agricultural industry, until it was banned in 1997 (5). The reported prevalence of VRE in hospitals has been low, but increasing rates (>10%) in stool and clinical samples were reported recently (6–9). In the United States, avoparcin was never approved for use in agriculture, and neither were any other glycopeptides; consequently, VRE have not been found in animals or healthy persons. However,

nosocomial VRE infection and transmission have occurred much more frequently in the United States. Recent reports have documented, in hospitalized patients, horizontal transfer of the *vanA* gene from vancomycin-resistant *E. faecalis* to methicillin-resistant *Staphylococcus aureus* (MRSA), creating MRSA with high-level resistance to vancomycin (10–13). Nosocomial spread of VRE may therefore create a reservoir of mobile resistance genes for other, more virulent, nosocomial pathogens. Without extensive control measures, large-scale emergence of vancomycin-resistant *S. aureus* (VRSA) may be the next stage in the global crisis of antimicrobial resistance.

The existence of VRE in different ecologic niches complicates the understanding of its epidemiology. Although previous molecular epidemiologic studies on limited numbers of strains suggested host specificity and overrepresentation of certain clones in hospital outbreaks (14,15), these studies did not elucidate the patterns of evolutionary descent among VRE. We determined the population structure of 411 VRE and vancomycin-susceptible *E. faecium* (VSE) isolates by using multilocus sequence typing (MLST), explored the evolutionary origin of epidemic isolates associated with documented hospital outbreaks and other isolates, and assessed the association with ampicillin resistance and the presence of a recently discovered putative pathogenicity island (PAI) in *E. faecium* (16).

Materials and Methods

The strain collection included 5 categories of VRE and VSE: 1) 96 animal surveillance (bison, calves, cats, dogs, ostriches, poultry, pigs, rodents) isolates (43 VRE, 53 VSE) from 7 countries in Africa and Europe; 2) 57 epidemiologically unrelated community surveillance isolates (20 VRE, 37 VSE) from nonhospitalized persons from 7 countries in Australia and Europe; 3) 64 epidemiologically unrelated surveillance (fecal) isolates (45 VRE, 19 VSE) from hospitalized patients not linked to hospital outbreaks from 9 countries in Australia, Europe, and North and South America; 4) 162 epidemiologically unrelated hospital isolates (43 VRE, 118 VSE, 1 not determined) from clinical specimens (blood, pus, and urine) from 17 countries in Africa, Australia, Europe, and North and South America; and 5) 1 strain from each of 32 different documented hospital outbreaks (28 VRE, 4 VSE) in 10 countries in Australia, Europe, and North and South America (W. Grubb and D. Jonas, pers. comm.; 15,17–23).

We determined vancomycin susceptibilities for 410 isolates and ampicillin susceptibilities for 381 isolates by using standard agar dilution methods according to NCCLS guidelines. Isolates with MIC ≥16 μg/mL for ampicillin and

≥8 μg/mL for vancomycin were considered to be resistant. In total, 394 strains were screened for the *esp* gene with primer sets and amplification conditions described previously (24). Independent and combined effects of virulence and resistance markers on the abundance of complex-17 were estimated by using multiple logistic regression analysis (Stata 7.0, StataCorp LP, College Station, TX, USA).

MLST was carried out with a standard set of primers that amplify the 7 genes included in the *E. faecium* MLST scheme (14). Information on these loci, the latest set of primers, amplification conditions, and details of all isolates are available on the MLST Web site (http://efaecium.mlst.net).

The eBURST program was used to assess the genetic relationships of genotypes, to assign isolates to genetic complexes, and to study patterns of evolutionary descent of isolates within a complex (25). Complexes were identified by using the stringent (6/7 shared alleles) group definition with 1,000 bootstrap replicates. The BLAND program was used to examine the relationship between pairwise allelic differences and nucleotide sequence differences (26). If genetic diversity in *E. faecium* is mainly the result of accumulated point mutations, then recently diverged strains will have a high level of similarity in both their allelic profiles as well as in the nucleotide sequence of the nonidentical alleles, which results in a positive correlation between the number of nucleotide differences in nonidentical alleles and the number of allelic differences. However, such a trend will be absent when recombination plays an important role in generating the genetic diversity, since nonidentical alleles of closely related isolates can differ at multiple nucleotide sites.

To assess the effect of recombination on the population structure of *E. faecium* in more detail, the topologies of the 7 MLST gene trees were compared by using the Shimodaira-Hasegawa test (27). Briefly, maximum likelihood trees for each MLST gene were obtained under a general time-reversible model, with a proportion of invariant sites and rate heterogeneity among sites assuming a discrete gamma distribution with 8 categories (GTR+I+Γ model). PAUP* 4.0b10 was used to obtain the maximum likelihood trees by using a neighbor-joining starting tree followed by tree-bisection reconnection branch swapping (28). For a given gene, the Shimodaira-Hasegawa test compares the difference in log likelihoods of competing tree topologies. A null distribution of differences in log likelihoods was obtained by 1,000 replicates of nonparametric bootstrapping of reestimated log likelihoods. We conducted 107 Shimodaira-Hasegawa tests for each MLST gene by comparing the 7 MLST gene trees and 100 random trees separately generated for each of the MLST genes. In a clonal population, the different MLST housekeeping genes have similar tree topologies, but with

recombination, the different genes may have different tree topologies that may fit random trees better.

Associations between ampicillin resistance, presence of a novel putative *E. faecium* PAI, and genetic clustering in complex-17 were described by linear logistic regression models: log odds = $b_0 + b_1x_1 + b_2x_2 + b_3x_3 + \ldots + b_ix_i$. Log odds denotes the natural logarithm of the proportion of samples from an epidemiologic group belonging to complex-17, b_i denotes the parameter estimated by maximum likelihood methods and x_i the level of exposure, e.g., 0 and 1 for ampicillin resistance, vancomycin resistance, and the presence of PAI and 0–4 for the epidemiologic source of isolates: animal surveillance, community surveillance, hospital surveillance, clinical sample, and hospital outbreak, respectively.

Table 1. Frequency of ampicillin and glycopeptide resistance, the presence of the pathogenicity island (PAI), and log odds of all complex-17 and non–complex-17

	Genetic and phenotypic features*														
Epidemiologic source	Complex-17		Other†		Complex-17		Other†		Complex-17		Other†		Complex-17‡	Other†‡	Log odds§
	AmR	AmS	AmR	AmS	PAI+	PAI–	PAI+	PAI–	VanR	VanS	VanR	VanS			
Animal surveillance (n = 96), %	1	0	2	93	0	1	0	94	0	1	43	52	1 [1]	95 [99]	–4.55
Community surveillance (n = 57), %	0	0	1	46	3	0	0	47	3	0	17	37	3 [5]	54 [95]	–2.89
Hospital surveillance (n = 64), %	14	0	7	40	7	8	0	49	13	2	32	17	15 [23]	49 [77]	–1.18
Clinical (n = 162), %	85	2	13	47	47	47	4	57	21	73	22	45	95 [59]	67 [51	0.35
Hospital outbreak (n = 32), %	26	0	3	1	20	6	3	1	24	4	4	0	28 [88]	4 [12]	1.95

*Ampicillin resistant (AmR) or susceptible (AmS) not determined in 30 isolates, PAI present (PAI+) or absent (PAI–) not determined in 17 isolates, vancomycin resistant (VanR) or susceptible (VanS) not determined in 1 isolate.

†Not belonging to complex-17.

‡Numbers in brackets refer to the percentage of isolates that belong to the complex.

§The natural logarithm of the proportion of samples from an epidemiologic source belonging to complex-17.

Results

Identification of Clonal Lineages

MLST of 411 *E. faecium* isolates resulted in 175 different sequence types (ST). Clustering these types with the eBURST algorithm (25) showed 1 large complex of genetically related types. ST-22 was the primary founder; 3 minor complexes had ST-1, -69, and -94 as primary founders; 6 complexes had only 2 or 3 STs; and 57 singletons were not linked to the aforementioned complexes (Figure 1). Within

complex-22, ST-17 represents an important secondary founder of a distinct branch designated complex-17.

Selective Advantage of the Successful Hospital-adapted Complex-17

In all, 142 of 411 isolates belonged to complex-17, with a gradual increase in proportion among animal isolates (1/96), human community isolates (3/57), human hospital surveillance isolates (15/64), and human clinical isolates (95/162), to hospital-outbreak isolates (28/32) (Table 1). Ampicillin resistance, presence of the *E. faecium* PAI (16), and genetic clustering in complex-17 were strongly associated (Table 2).

When controlling for individual and combined effects of ampicillin resistance, presence of PAI, and vancomycin resistance, we can show that 1) the loglinear assumption holds for all effect parameters, and linear models describe the observed frequencies without substantial loss of goodness of fit; 2) individual

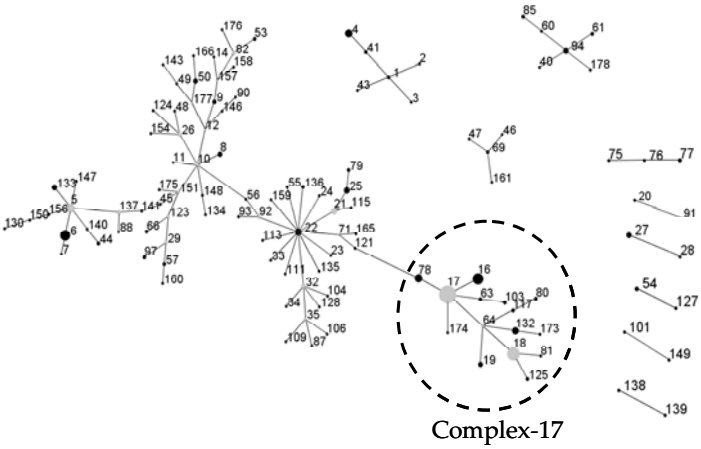

Figure 1. Clustering of 175 sequence types representing 411 isolates with eBURST (25). This algorithm identifies the founder of a complex or genogroup of related sequence types (ST) and subsequent patterns of evolutionary descent. The primary founder, indicated in dark grey, of a complex is defined as the ST with the largest number of single locus variants (SLVs). Larger complexes may contain secondary founders of additional lineages that have a number of SLVs of their own. These secondary founders are indicated in light grey. Numbers correspond to ST numbers. The area of each circle corresponds to the number of isolates of the ST. All complexes (major and minor) are shown. In addition, 57 STs did not group into any of the complexes and were considered singletons(STs 13, 15, 30, 31, 36, 37, 38, 39, 42, 51, 52, 58, 59, 62, 65, 67, 68, 70, 72, 73, 74, 83, 84, 86, 89, 95, 96, 98, 99, 100, 102, 105, 107, 108, 110, 112, 114, 116, 118, 126, 129, 131, 142, 144, 145, 152, 153, 155, 162, 163, 164, 167, 168, 169, 170, 171, 172). The"epidemic" genetic complex-17 derived from secondary founder ST-17 is indicated. A measurement of statistical confidence in each of the assigned primary founders is made by a bootstrap resampling procedure (25).The predicted primary founders of the complexes 22, 94, 1, and 69 have a bootstrap value of 73%, 84%, 85%, and 59%, respectively.

genetic markers exert an independent and multiplicative effect; and 3) all genetic markers combined explain ≈48% of the category-specific abundance of complex-17 (Figure 2). The effect of vancomycin resistance did not increase the explanatory value of the model, owing to the fact that determinants for vancomycin resistance could be found in equal proportions within and outside of complex-17, likely a result of widespread horizontal transfer of *vanA* (Table 1). This finding suggests that the epidemiologic success of descendants of ST-17 that results in clinical infections and hospital epidemics was at least partly related to antimicrobial resistance and the presence of putative virulence genes. The fact that 126 of 128 isolates of complex-17 were resistant to ampicillin and only 77 of 139 isolates of complex-17 contain PAI (Table 1) suggests that *E. faecium* acquired ampicillin resistance first, which resulted in a selective advantage in hospitals, followed by the acquisition of PAI, which further facilitated transmission.

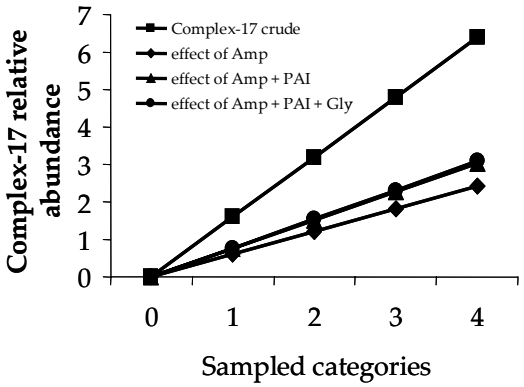

Figure. 2. Relative abundance of complex-17 in various sampled categories and proportion increase explained by combined effect of 3 parameters. 0, animal surveillance samples; 1, human community surveillance samples; 2, human hospitalised patients samples; 3, human clinical samples; 4, hospital outbreak samples; amp, ampicillin resistance; PAI, pathogenicity island; gly, glycopeptide resistance.

Estimates of Recombination

To assess the effect of recombination on the population structure of *E. faecium*, we estimated whether single locus variants (SLVs) from the presumed founders of complex-1, -17, -22, -69, and -94 have arisen by point mutations or by recombination (Table 3). Of all allelic differences between ancestor-SLV pairs (n = 30), 22 (4 in complex-17) included >1 nucleotide, were found in multiple clonal complexes, and thus were most likely a result of recombination. Eight allelic differences (2 in complex-17) included only a single nucleotide change, were unique within the dataset, and thus were most likely a result of mutation. Therefore, most alleles of SLVs in complex-17 and in the other complexes have arisen by recombination in the initial stages of diversification rather than by de

Table 2. Parameter estimates by using a logistic regression model*

	Parameter estimates					
Regression lines	b_0	b_{amp}	b_{PAI}	b_{gly}	b_{epi}	p value
Complex-17 crude	–4.44	0	0	0	1.6	0.000
Corrected for amp	–6.61	5.38	0	0	1	0.000
Corrected for PAI	–6.29	5.08	1.06	0	0.84	0.038
Corrected for gly	–6.07	5.12	1.01	–0.45	0.83	0.316

*Amp, ampicillin resistance; PAI, presence of the *Enterococcus faecium* pathogenicity island; gly, glycopeptide resistance; epi, epidemiologic source (animal surveillance, community surveillance, hospital surveillance, clinical sample, hospital outbreak).

novo point mutation. An important role for recombination in genetic diversification in *E. faecium* was confirmed by the lack of a positive trend between the number of nucleotide differences in nonidentical alleles and the number of allelic differences (Figure 3) (26). The finding of high average numbers (≥4) of nucleotide differences in the nonidentical alleles of SLVs in the total *E. faecium* population as well as in complex-17 also points towards frequent recombination.

The degree of phylogenetic congruence between the 7 MLST genes was examined in a set of 24 diverse STs. These 24 STs were separated from each other by a linkage distance of >0.4 on a UPGMA (unweighted pair-group method with arithmetic mean) tree constructed from the pairwise comparisons of their allelic profiles (data not shown) and included the primary founders of CC1 (ST1), CC22 (ST22), CC69 (ST69), and CC94 (ST94); secondary founders of important subgroups complex-5 (ST5) and complex-17 (ST17); 1 ST (ST76) belonging to a small complex of 3 STs; and 17 singletons (STs 15, 38, 39, 54, 67, 74, 83, 84, 89, 96, 98, 99, 101, 107, 118, 142, 163). The results of the congruence analysis presented in Table 4 show that 25 (60%) of 42 of the pairwise comparisons of the 7 MLST loci were incongruent. Of the 7 genes, *atpA* is the most incongruent. This analysis confirms that recombination played a substantial role in the evolution of *E. faecium*.

Discussion

Nosocomial VRE, which rapidly emerged in the United States in the 1990s after their initial discovery in Europe, are found in increasing rates in hospitals in Europe, Asia, and South America (5–7,9,23,29,30). The data presented in this study show that most of these hospital-derived VRE are part of a single clonal lineage. This lineage, designated complex-17 after its presumed founder ST-17, represents most hospital outbreak and clinical isolates, apparently because it successfully adapted to hospital environments.

The >400 strains analyzed in this study were selected from a large representative collection of 2,000 *E. faecium* isolates. A wide variety of sources were used as selection criteria: hospital-associated outbreaks; clinical samples and

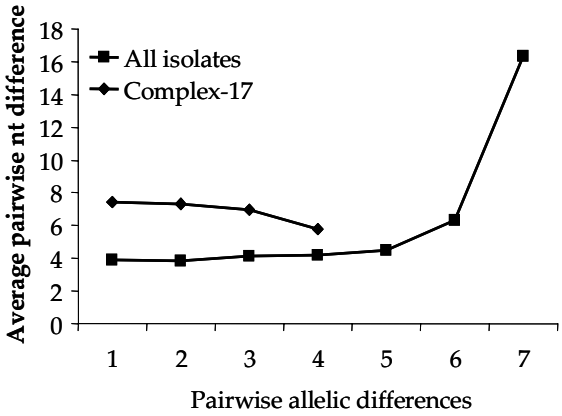

Figure 3. Sequence diversity versus allelic diversity. The average number of nucleotide (nt) differences in non-identical alleles for all pairwise comparisons of the 178 *Enterococcus faecium* sequence types (STs) and the 15 STs belonging to complex-17 was calculated separately for allelic profiles that differ in 1-7 alleles. This computation shows no positive correlation between the number of nucleotide differences and allelic differences, which suggests that recombination has played an important role in the genetic diversification of *E. faecium*, including the STs that constitute complex-17.

stool samples from hospitalized patients, healthy persons, and animals; and a wide geographic distribution (21 countries on 5 continents).

Complex-17 probably evolved from the primary *E. faecium* ancestor ST-22 through a combination of mutation and recombination. The following observations suggest that recombination has been especially important in the genetic diversification of the *E. faecium* population: 1) within clonal complexes, most SLVs (73%) have arisen by recombination rather than point mutations; 2) no positive correlation exists between the degree of allelic diversity and the number of nucleotide differences in nonidentical alleles; and 3) most (60%) of the comparisons of MLST gene tree topologies were incongruent.

Exploitation of a novel ecologic niche as hospital settings by *E. faecium* ST-17 often starts with adaptive changes (31). On the basis of our findings, we postulate that ST-17 acquired ampicillin resistance and a novel putative PAI. This amplifying selective process in which variants with a selective advantage can more easily acquire additional adaptive mechanisms has been called "genetic capitalism" (32). After successfully exploiting the hospital environment, ST-17 increased in frequency to become the dominant clone. Genetic diversification over time finally resulted in a meroclone, complex-17, of highly related genotypes, fully adapted as a nosocomial pathogen, that has spread globally (Figure 4). In addition, *E. faecium* STs, predominantly ST-78, belonging to complex-17, have recently also been found in the Republic of South Korea (K.S. Ko and J.-H. Song, pers. comm.). Considering the short period in which multiresistant *E. faecium*

Table 3. Variant alleles of single locus variants (SLVs) within 5 genetic complexes*

ST of ancestor	ST of SLV	Variant locus	Ancestral allele	SLV allele	No. nucleotide differences (amino acid change)
17	64	*atpA*	1	7	4
17	117	*atpA*	1	9	20
17	78	*atpA*	1	15	22
17	16	*ddl*	1	2	1
17	63	*purK*	1	21	1 (C-Y)†
17	174	*purK*	1	29	1†
22	32	*atpA*	2	3	2
22	21	*atpA*	2	9	18
22	92	*atpA*	2	5	19
22	71	*atpA*	2	15	20
22	135	*atpA*	2	27	18
22	159	*atpA*	2	30	1†
22	113	*atpA*	2	26	19
22	55	*ddl*	3	1	6
22	111	*gdh*	1	6	1
22	24	*purK*	2	7	3
22	136	*purK*	2	26	1 (H-Y)†
22	33	*pstS*	1	5	1 (L-V)†
22	23	*adk*	1	7	1†
1	43	*atpA*	8	3	16
1	41	*purK*	7	3	4
1	2	*gyd*	1	9	1 (Y-H)†
1	3	*pstS*	1	12	1 (Y-N)†
94	40	*atpA*	13	10	3
94	60	*gyd*	6	11	1
94	61	*pstS*	10	17	4
94	178	*pstS*	10	27	2
69	46	*atpA*	9	5	1
69	161	*atpA*	9	3	16
69	47	*adk*	6	5	4

*Genetic complexes 1, 17, 22, 69, and 94 were included in this analysis. ST, sequence type; C, cysteine; Y, tyrosine; H, histidine; L, leucine; V, valine; N, asparagine.

†Single nucleotide changes that are unique in the dataset and thus are due to mutation.

emerged as a nosocomial pathogen (33), complex-17 represents the first globally dispersed nosocomial-adapted clonal lineage. Despite the frequency of recombination events, clonal complex-17 is still detectable within the *E. faecium* population, which suggests that the emergence of this complex is relatively recent.

The existence of epidemic clones, even in recombining populations, is also seen in other bacterial species (34,35). However, the evolution of a single epidemic and clinically relevant genetic complex, as seen with *E. faecium*, differs from the evolution of other gram-positive pathogens like *Streptococcus pneumoniae*

Table 4. Summary of gene congruence analysis

Gene	No. incongruence genes by SH test*	Random trees†
adk	1 (*atpA*)	8 (*atpA*)
atpA	6 (*adk* , *ddl* , *gdh* , *gyd* , *pstS* , *purK*)	76 (*adk*)
ddl	6 (*adk* , *atpA* , *gdh* , *gyd* , *pstS* , *purK*)	8 (*atpA*)
gdh	1 (*atpA*)	1 (*atpA*)
gyd	2 (*adk* , *atpA*)	0 (*atpA*)
pstS	6 (*adk* , *atpA* , *ddl* , *gdh* , *gyd* , *purK*)	3 (*atpA*)
purK	3 (*atpA* , *adk* , *gyd*)	1 (*atpA*)

*Number of incongruent genes at the p<0.05 level based on a Shimodaira-Hasegawa (SH) test of tree topologies. The incongruent genes are in parentheses.

†Number of random tree topologies out of 100 random trees that are better fit to the gene tree from the most incongruent multilocus sequence typing (MLST) gene. The most incongruent MLST gene is given in parentheses.

and *Staphylococcus aureus*. In *S. pneumoniae*, pandemic clones such as ST81, ST90, and ST156 represent major invasive and multidrug-resistant isolates that have spread globally (36). The allelic profiles of these clones, however, are highly diverse, which suggests that they are genetically unrelated and do not constitute a single genetic lineage, as does *E. faecium*. Furthermore, the serotype of *S. pneumoniae* seems a more important marker of invasiveness than the overall genotype (37). In *S. aureus* isolates, major pandemic MRSA clones that are responsible for most hospital-acquired infections are found in multiple genetically unrelated lineages, though most previously identified pandemic clones are found in clonal complex 8 (38,39). Therefore, the genetic diversity of major epidemic clones as seen in *S. pneumoniae* or *S. aureus* may not have yet emerged in *E. faecium* epidemic populations.

Stress-inducing conditions in hospitals, such as antimicrobial drug use, may have favored the selection of an enterococcal subpopulation, complex-17, with enhanced antibacterial resistance, virulence, and ability to spread. Whether reducing antimicrobial selection pressure in hospitals will reestablish a susceptible and less transmissible enterococcal population is unknown and will at least partly depend on the relative fitness costs of sustaining antimicrobial resistance and virulence determinants in *E. faecium*. Furthermore, the hospital-adapted complex-17 has rapidly spread globally during the last 2 decades. Subsequent acquisition of *vanA*- or *vanB*- containing transposons by horizontal gene transfer resulted in VRE with pandemic potential. Rapid diagnosis of complex-17 strains based on multiple locus variable number of tandem repeat analysis (MLVA) may help control its spread (40). Whether this effort will be successful depends on the level of complex-17 endemicity in the hospital. In many European countries, a relatively large community reservoir of VRE exists, a result of the massive use of the antimicrobial drug avoparcin as a growth promoter, while in general the prevalence of hospital-adapted (complex-17) VRE is much lower. In such a setting, hospital transmission of isolates belonging to

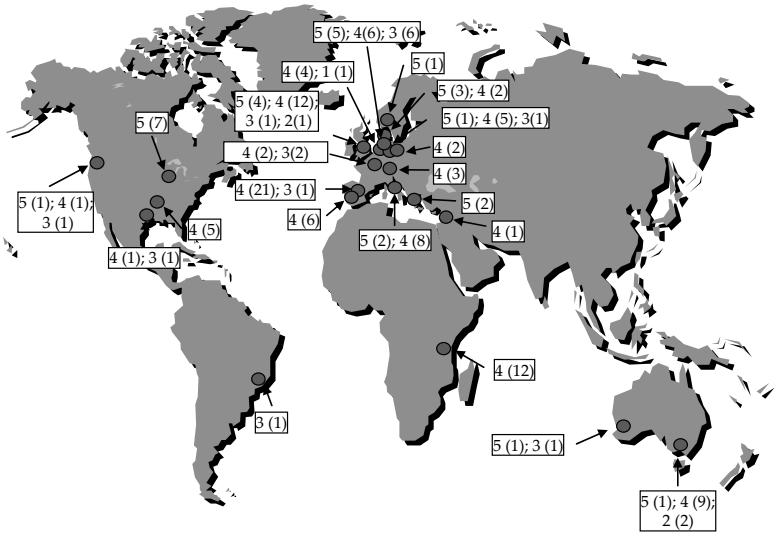

Figure 4. Global distribution of complex-17 isolates. Circles indicate cities where complex-17 isolates were recovered. Numbers indicate epidemiologic sources: 1, animal isolates; 2, human community surveillance isolates; 3, surveillance (feces) isolates from hospitalized patients; 4, human clinical isolates; 5, isolates from documented hospital outbreaks. Numbers of isolates are indicated in parentheses.

complex-17 can be halted by using a fast genotyping scheme like MLVA to discriminate between hospital-adapted (complex-17) and community *E. faecium* strains followed by strict infection control measures. The combination of infection control measures plus genotyping controlled an outbreak of VRE in a Dutch hospital (41).

Establishing nosocomial co-endemicity of VRE and MRSA will facilitate the horizontal transfer of *vanA*- or *vanB*-containing transposons, transforming MRSA into VRSA, with implications for patient care. Until now, 3 sporadic cases of *vanA*-induced VRSA have been reported in the United States in 2002 and 2004. Spread of multidrug-resistant *E. faecium* strains and their resistance genes will have serious implications for health care, and control efforts should focus on early detection of *E. faecium* isolates belonging to complex-17.

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Dr. Willems began this research at the National Institute for Public Heath and the Environment and continued at the University Medical Center Utrecht, where he is currently working. His research interests are the molecular epidemiology, population structure, and genetic evolution of multidrug-resistant nosocomial pathogens.

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Chapter 7

Ecological replacement of *Enterococcus faecalis* by multi-resistant clonal complex 17 *Enterococcus faecium*.

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Abstract

The proportion of enterococcal infections caused by ampicillin-resistant *Enterococcus faecium* (AREfm) in a European hospital increased from 2% in 1994 to 32% in 2005, with prevalence rates of AREfm endemicity of up to 35% in at least six hospital wards. Diabetes mellitus, three or more admissions in the preceding year, and use of β-lactams and fluoroquinolones, were all associated with AREfm colonisation. Of 217 AREfm isolates that were genotyped, 97% belonged to clonal complex 17 (CC17). This ecological change mimics events preceding the emergence of vancomycin-resistant *E. faecium* (VREF) in the USA and may presage the emergence of CC17 VREF in European hospitals.

Introduction

Molecular epidemiological studies of *Enterococcus faecium* have revealed the existence of host-specific genogroups, including an ampicillin-resistant genetic lineage, labelled clonal complex 17 (CC17), which are associated with nosocomial outbreaks and infections in five continents (1). In European hospitals, rates of infection with vancomycin-resistant *E. faecium* have been rising since the year 2000 (EARSS Annual Report 2004; http://www.rivm.nl/earss), suggesting that the emergence of vancomycin-resistant *E. faecium* in Europe may be following the pattern observed in the USA, but with a 10-year delay. The emergence of vancomycin-resistant *E. faecium* in the USA was preceded by the emergence of ampicillin resistance in *E. faecium* (2,3).

Material and methodes

Stimulated by an increase in ampicillin-resistant *E. faecium* (AREfm) bloodstream infections during 2003, the present study analysed trends in enterococcal infection and colonisation at the University Medical Centre, Utrecht, The Netherlands (1042 beds). The prevalence of invasive enterococcal infections was assessed retrospectively using microbiological data for 1994–2005. Invasive infections were defined as infectious episodes with enterococci isolated from normally sterile specimens, e.g., blood, abdominal fluid, intravascular catheter tips, cerebrospinal fluid, pus and wound specimens. Enterococci isolated from urine were not considered to represent invasive infections. Yearly proportions of *Enterococcus faecalis* and *E. faecium* among enterococcal bloodstream infections were determined on the basis of the first 20 enterococcal blood culture isolates (one per patient) per year. The intestinal AREfm reservoir was measured by monthly point-prevalence studies between August 2005 and January 2006 in

Table 1. Risk-factor analysis for colonization with ampicillin-resistant *Enterococcus faecium*

Variable	Cases(%) (n=43)	Controls(%) (n=93)	OR	95%CI	p value[a]
Univariate analysis					
Demographic and clinical data					
Medical speciality (nephrology)	72.1	39.8	3.91	1.78-8.57	<0.001
Age, mean years (±SD)	57.4 (±14.4)	54.4 (±16.0)			0.29[b]
Male gender	51.2	43.0	1.39	0.67-2.87	0.38
Length of stay, median of days (range)	10 (1-78)	7 (1-55)			0.19[c]
Number of readmissions					
in preceding year					
0	32.6	59.1			<0.001
1-2	37.2	35.5			
≥3	30.2	5.4			
CAPD	27.9	9.7	3.613	1.39-9.41	0.006
Haemodialysis	25.6	9.7	3.208	1.22-8.47	0.02
Kidney transplantation	27.9	14	2.382	0.98-5.79	0.05
Recent surgery	34.9	24.7	1.630	0.74-3.57	0.22
Malignancy	4.7	8.7			0.25
Immunecompromised state	50	27.2	2.680	1.25-5.73	0.01
Systemic use of corticosteroids	48.8	29	2.333	1.11-4.93	0.03
Cirrhosis of the liver	9.3	5.4	1.805	0.46-7.09	0.39
Crohn's disease	2.3	5.4	.419	0.05-3.7	0.42
Colitis ulcerosa	0	2.2			0.33
Diabetes mellitus	23.3	8.6	3.220	1.17-8.87	0.02
Antibioticusage	76.7	49.5	3.372	1.49-7.63	0.003
β-Lactams	65.1	37.6	3.09	1.46-6.58	0.004
Co-trimoxazol	25.6	8.6	3.652	1.35-9.9	0.008
Macrolides	4.7	3.2	1.463	0.24-9.1	0.68
Vancomycin	2.3	3.2	.714	0.07-7.07	0.77
Quinolones	18.6	5.4	4.023	1.23-13.15	0.02
Aminoglycosides	4.7	10.8	.405	0.09-1.93	0.24
Multivariate analysis[d]					
CAPD			2.75	0.82-9.20	0.10
Haemodialysis			3.44	0.96-12.36	0.06
Kidney transplantation			0.44	0.09–2.24	0.32
Immunecompromised state			1.14	0.22-5.95	0.88
Systemic use of corticosteroids			5.68	1.18-27.31	0.30
Diabetes mellitus			8.59	2.08-35.44	0.003
β-Lactams			2.97	1.09-8.09	0.03
Co-trimoxazol			2.38	0.58-9.71	0.23
Quinolones			5.23	1.22-22.48	0.03
Number of readmissions					
in preceding year					
0			1	Reference	0.001
1-2			1.75	0.62–4.91	0.29
≥3			14.84	3.44–64.10	<0.001

[a]Chi-square test.

[b]*t* -test

[c]Mann-Whitney test.

[d]Logistic regression on variables with p<0.100.

CAPD, continuous ambulatory peritoneal dialysis

seven hospital wards (haematology, 21 beds; gastroenterology/nephrology, 25 beds; adult intensive care unit (ICU), 24 beds; paediatric ICU, 47 beds; geriatrics, 15 beds; general surgery, 30 beds; and dermatology, 12 beds). The AREfm reservoir in the community was investigated using faecal samples from 650 outpatients with abdominal discomfort who visited general practitioners in the Utrecht region during 2004.

Risk-factors for colonisation with AREfm were determined using clinical and demographical data for patients in the mixed gastroenterology/nephrology ward. Statistical analysis was performed with SPSS v.12.0.1 (SPSS Inc., Chicago, IL, USA). The clinical impact of AREfm was determined by analysis of clinical, demographical and outcome data for all patients with an invasive AREfm infection between May 2001 and November 2005.

Enterococcosel enrichment broth and agar plates (Becton Dickinson, Cockeysville, MD, USA), supplemented with aztreonam 75 mg/L and amoxycillin 16 mg/L, were used to obtain isolates of AREfm. Resistance was confirmed by amoxycillin Etests (AB Biodisk, Solna, Sweden). A species-specific multiplex PCR, based on the *ddl* gene of *E. faecalis* and *E. faecium*, was used for speciation (4). Susceptibilities to ampicillin and imipenem were determined by inoculation of

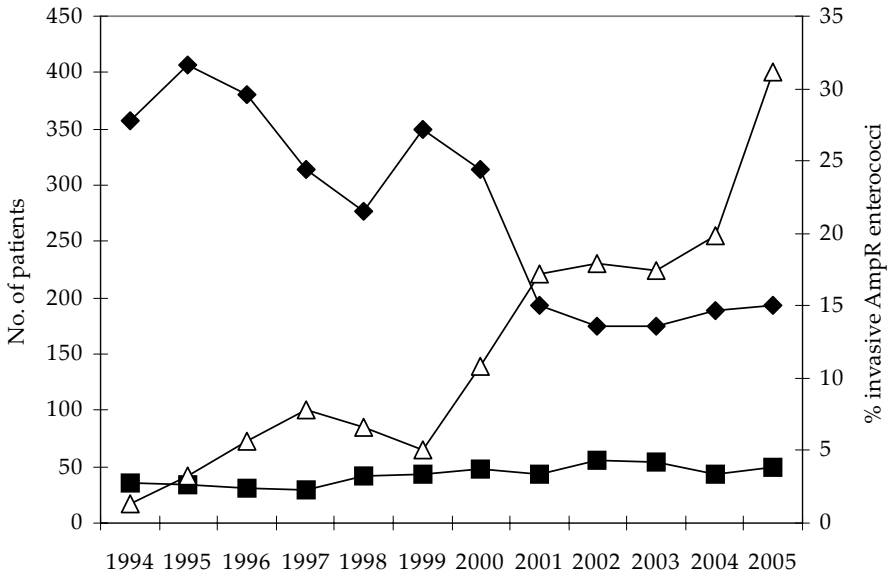

Figure. 1. Invasive enterococcal infections, 1994–2005. ■, No. of patients with enterococcal bloodstream infections.♦, No. of patients with other invasive enterococcal infection. Δ, Percentage of invasive ampicillin-resistant(AmpR) enterococci among the total number of invasive enterococci.

Mueller–Hinton agar containing ampicillin 8 mg/mL and imipenem 16 mg/mL, according to CLSI (formerly NCCLS) guidelines. Isolates were genotyped using multiple locus variable number tandem repeat analysis (MLVA), which is based on variations in the number of tandem repeats at six different loci (5). MLVA profiles were analysed using BioNumerics v.4.00 (Applied Maths, St-Martins-Latem, Belgium).

Results

The overall number of patients with invasive enterococcal infections decreased from 393 in 1994 to 243 in 2005, but proportions of ampicillin-resistant enterococcal infections increased from 2% in 1994 to 32% in 2005 ($p < 0.001$) (Figure. 1). *E. faecium* increased from 3% of enterococcal bloodstream infections in 1994–1996 to 30% in 2003–2005 ($p < 0.001$), and 75% of *E. faecium* blood culture isolates were resistant to both ampicillin and imipenem, compared with 0% for *E. faecalis* ($p < 0.001$).

Point-prevalence studies revealed carriage rates ranging from 0% in dermatology to 10.3% in the paediatric ICU, 29% in gastroenterology/nephrology, 29.2% in the adult ICU, 34.6% in haematology and 34.8% in geriatrics. AREfm isolates were obtained from 19 (2.9%) of 650 community-derived faecal samples. No data were available concerning previous hospitalisation or antibiotic use for these patients.

Diabetes mellitus (OR 8.59, 95% CI 2.08–35.44), three or more admissions in the preceding year (OR 14.84, 95% CI 3.44–64.10) and use of β-lactams (OR 2.97, 95% CI 1.09–8.09) or quinolones (OR 5.23, 95% CI 1.22–22.48) were associated independently with AREfm colonisation (Table 1).

Between May 2001 and November 2005, 167 patients had an AREfm infection, with blood (n = 53) and pus (n = 30) as the predominant samples. Overall, 154 (92%) of 167 patients had received antibiotics before developing an infection with AREfm. Nineteen of 20 haematology patients with AREfm infection had bacteraemia. Haematology patients in the studied hospital receive ciprofloxacin prophylaxis during prolonged granulocytopenia, and imipenem is the empirical antibiotic regimen for granulocytopenic fever. During 2002–2005, 7% (n = 12) of all episodes of bacteraemia during granulocytopenia were caused by AREfm. The average period between obtaining blood cultures and commencing appropriate antimicrobial therapy (i.e., vancomycin) was 2 days. Of 167 patients with invasive AREfm infections, 58 (35%) died during hospitalisation, with an average period of 15 days (0–105 days) between identification of the AREfm infection and death.

MLVA typing of 217 AREfm isolates revealed 40 different MLVA types, of which 211 (97%) belonged to CC17 (data not shown). A gradual increase in

infection and colonisation episodes with CC17 *E. faecium* has occurred in this hospital in recent years, with bacteraemia accounting for 32% of all infections, an overall mortality rate of 35%, and CC17 infections being most prevalent among high-risk patients (i.e., transplant or ICU patients).

Discussion

E. faecium CC17 consists mainly of clinical isolates and isolates associated with hospital outbreaks of vancomycin-resistant enterococci (VRE) (1). Strains colonising healthy individuals and animals cluster, almost without exception, outside this complex. It has been postulated that specific adaptations to the hospital environment that facilitate efficient spread are the reasons for the success of this pathogen (1). However, the spread of multiple CC17 subclones, without an existing community reservoir, can only be explained by cross-transmission and selective antibiotic pressure. Increasing rates of infection with AREfm present a therapeutic dilemma, as amoxycillin has been the preferred antibiotic for invasive enterococcal infections. In bone marrow transplant patients (for whom imipenem was first-choice therapy for granulocytopenic fever), surveillance for AREfm carriage has now been implemented and vancomycin has been added to imipenem for the treatment of granulocytopenic fever in AREfm carriers.

A similar rise of AREfm may have preceded the nationwide nosocomial epidemic of VRE in the USA. Three longitudinal microbiology-based studies in the USA reported changes in *E. faecalis*/*E. faecium* ratios in hospital infections (6–8). High prevalence and nosocomial spread of AREfm have also been reported, albeit sporadically, in European countries (9–14). The emergence of AREfm may presage the emergence of VRE, following horizontal transfer of vancomycin resistance genes into AREfm (15,16). In Europe, the prevalence of VRE carriage among healthy individuals decreased to 3% after the ban on the use of avoparcin in the agricultural industry in 1996 (17,18). However, the prevalence of VRE in a cohort of non-hospitalised patients in the Utrecht region during the year 2000 was still 2%(19), which represents a relatively abundant pool of vancomycin resistance genes in the community.

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Chapter 8

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

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Manuscript in preparation

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).

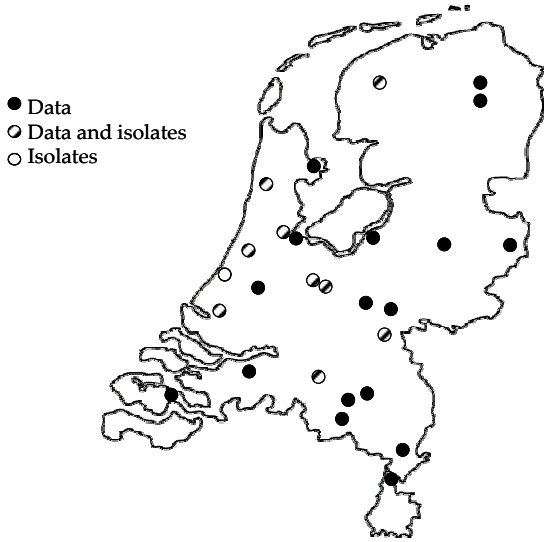

Figure 1. Distribution of contributing hospitals over the Netherlands. Solid dots represent hospitals, which provided data only; hatched dots hospitals, which provided data and bloodstream isolates and open dot, one hospital, which provided bloodstream isolates only

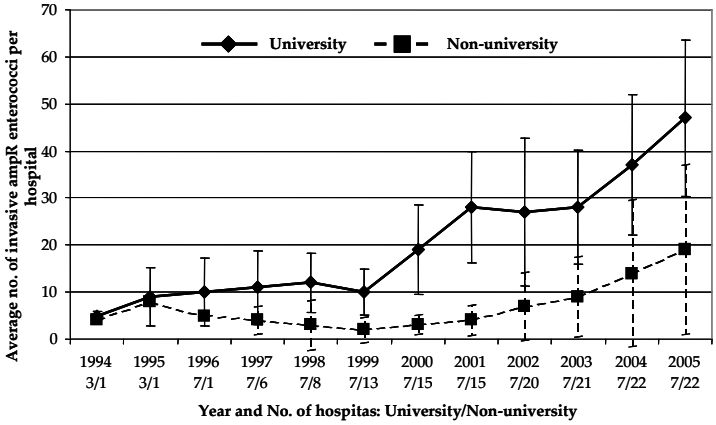

Figure 2. Average annual numbers of invasive ampR enterococci per hospital. Error bars denote standard deviations. Comparison of university- and non-university hospitals. For each year numbers of hospitals, which provided data are indicated.

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).

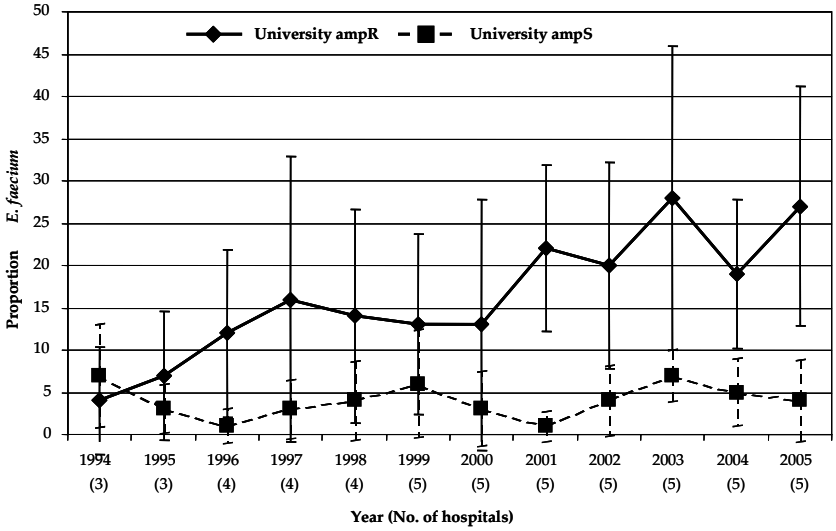

Figure 3. Average annual proportions ampicillin resistant (ampR) and ampicillin susceptible (ampS) *E. faecium* among enterococcal bloodstream isolates from university hospitals. Error bars denote standard deviations. For each year numbers of hospitals, which provided isolates are indicated.

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.

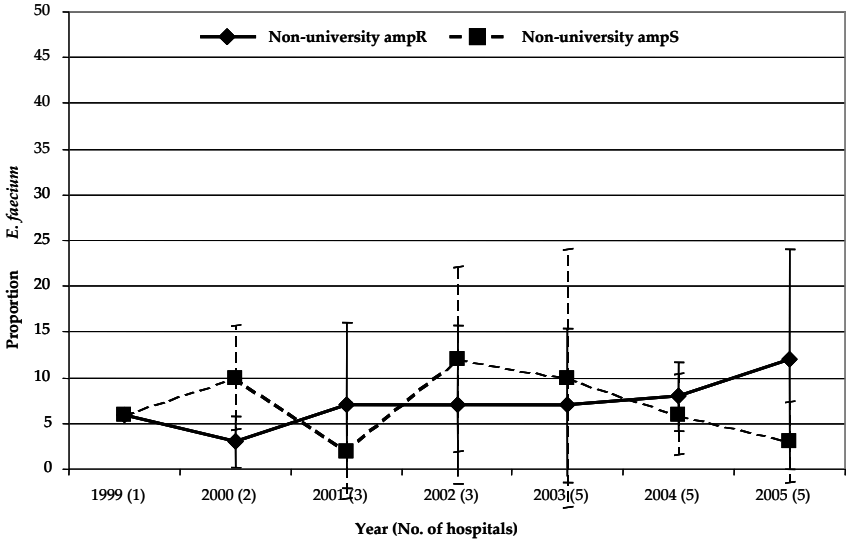

Figure 4. Average annual proportions ampicillin resistant (ampR) and ampicillin susceptible (ampS) *E. faecium* among enterococcal bloodstream isolates from non-university hospitals. Error bars denote standard deviations. For each year numbers of hospitals, which provided isolates are indicated.

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

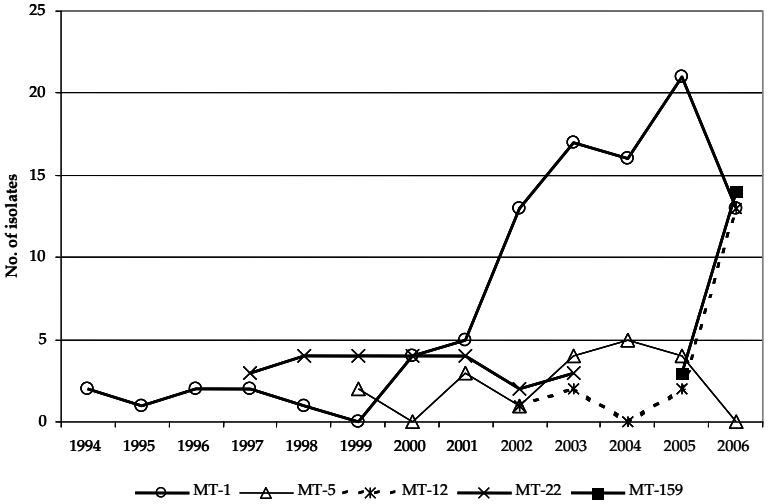

Figure 5. Annual distribution of five predominant MLVA types (MT).

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).

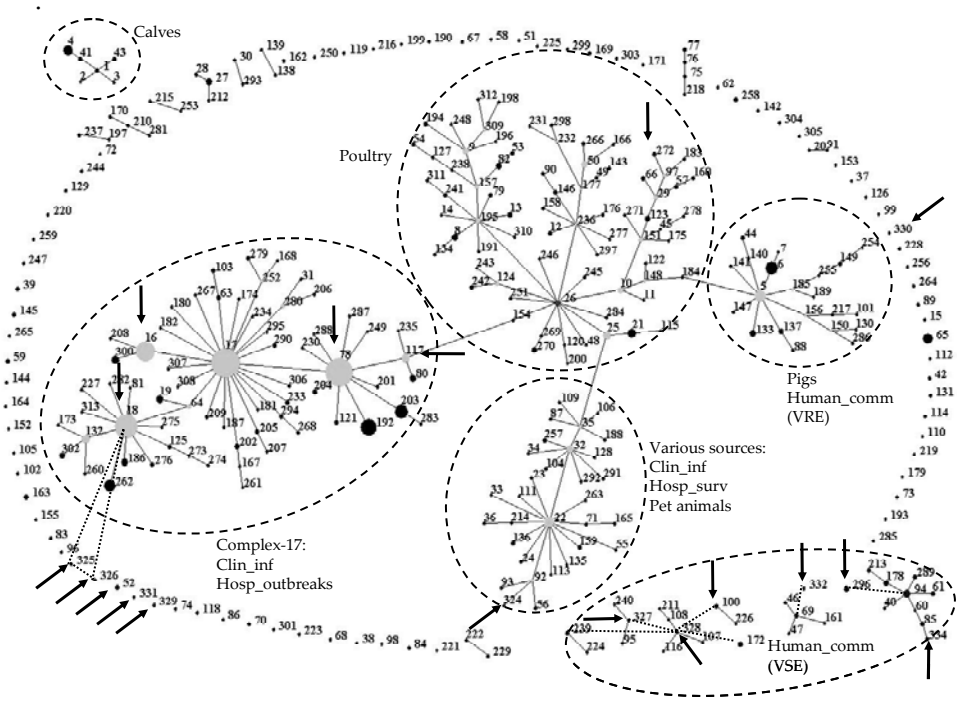

Figure 6. eBURST clustering of 18 MLST profiles, indicated with an arrow, representing 27 isolates from current study, with 313 MLST profiles representing 855 *E. faecium* isolates from the database (www.mlst.net). Each ST is represented as a node, the relative size of each node is indicative for their 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.

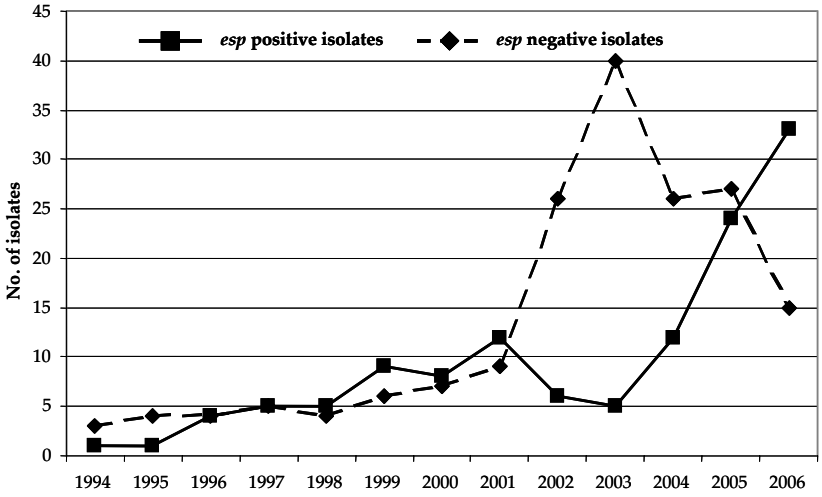

Figure 7. Comparison of the annual distribution of *esp* positive and negative isolates.

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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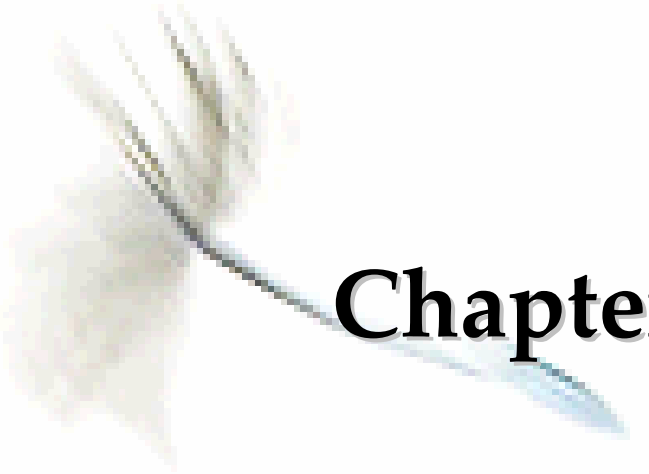

Chapter 9

General discussion

For many years *Enterococcus faecium* was considered a commensal of the digestive tract, which could sometimes cause opportunistic infections in severely ill patients, while *Enterococcus faecalis* was much more prevalent causing 80-90% of enterococcal infection. In the early 1980s, initial reports of increased infection rates and outbreaks due to ampicillin resistant *E. faecium* were published, which was followed by increased infection rates and outbreaks due to glycopeptide resistant enterococci (both *E. faecium* and *E. faecalis*) in the late 1980s. Based on molecular typing of a small number of vancomycin resistant *E. faecium* (VREF) isolates, clustering of hospital related isolates was observed. These strains were characterized by ampicillin and quinolone resistance. In addition, the presence of the variant *esp* gene was strongly associated with these outbreak strains. In 2001, Esp was described as a putative virulence factor in *E. faecalis*, and 1 year later Shankar and coworkers demonstrated that in *E. faecalis esp* was contained on a so-called pathogenicity island (PAI).

This thesis describes the molecular epidemiological studies of *E. faecium*, from a hospital wide (University Medical Center Utrecht, (UMCU)), nationwide (the Netherlands) and global perspective. Furthermore, a rapid identification scheme for enterococci and a rapid molecular typing scheme for *E. faecium* were developed. Finally, the chromosomal region encompassing the *E. faecium esp* gene was characterized.

The identification of enterococci is subject to a lot of controversies. Especially the discrimination between *E. faecium* and the low level vancomycin intrinsic resistant *E. casseliflavus* and *E. gallinarum* isolates is problematic. Rapid and correct identification of enterococci is a prerequisite to take appropriate infection control measures. The evaluation of different phenotypic methods described in chapter 2 revealed that the 100% reliable method doesn't exist as a result of a-typical reacting enterococci. Even with the proposed rapid identification scheme (4 hours), based on five phenotypic tests a few a-typical reacting isolates were found resulting in an accuracy of 92%. Raman spectroscopy, a phenotypic identification method under development, appeared to be a promising tool for the rapid identification of enterococci, but the reference database needs to be extended with other enterococcal species before the reliability of Raman spectroscopy in the identification of enterococci can be determined.

Molecular typing schemes based on multiple loci dispersed over the chromosome, like multi locus sequence typing (MLST) and multiple locus variable number tandem repeat analysis (MLVA), can be used to confirm species identification, but more importantly also to determine the genetic relatedness of

bacterial isolates. In chapter 3, MLVA was introduced as a novel rapid and cheap typing scheme for *E. faecium*, which was based on variation in numbers of tandem-repeats.

Most MLVA typing schemes developed for other bacterial species are based on small repeat units e.g. 12 bp, which need special equipment like a DNA sequencer for analysis of band sizes. Variation in numbers of small repeats is thought to result from "mistakes" by the DNA polymerase during replication, the so-called slip strand mispairing mechanism. Our goal was to develop a rapid typing method. Therefore large repeat units, easily detectable on agarose gels without special equipment, were chosen. Due to the size of the repeat units (between 121 and 279 bp) in the *E. faecium* MLVA typing scheme, it is unlikely that slip strand mispairing is the underlying mechanism in variation in numbers of repeats. Variation in large repeat units is probably a result of recombination events. The fact that recombination is an important mechanism for genetic diversification of *E. faecium* was also observed by MLST analysis on a set of 411 isolates (chapter 6).

Although a correlation in variation in tandem repeats and mutation/recombination in housekeeping genes seems unlikely, surprisingly, MLVA was highly congruent to MLST in recognizing the MLST-based hospital adapted CC17 isolates, formerly designated the C1- lineage (chapter 3). eBURST clustering of 127 MLVA profiles predicted MT-1 as the primary founder of this cluster. Despite the high congruence several different STs (predominantly ST17) were found among MT-1 isolates. But, vice versa, different MTs were found among ST17 isolates as well. Therefore, one could conclude that although the clock-speed of these genetic events is not known, they might be different for tandem repeats and housekeeping gene variation.

While MLVA is a relative new technique, this typing scheme was compared with Pulsed Field Gel Electrophoresis (PFGE), which is still considered the "gold standard" typing method in infection control programs in hospitals (chapter 4). There are, however, several pitfalls related to PFGE typing of *E. faecium* isolates. In *E. faecium* a high degree of genetic rearrangements due to the presence of mobile elements has been observed, resulting in DNA banding pattern polymorphisms and affecting PFGE pattern stability. Therefore, PFGE of *E. faecium* is only suitable to be used for short-term and local epidemiology like outbreak situations in a single hospital. For long-term or global epidemiology evolutionary related isolates are no longer recognized due to lack of stability of PFGE banding patterns. Furthermore, PFGE typing is labor-intensive, lacks standardized methods and criteria for interpretation of banding patterns, and is, therefore, not suitable for interlaboratory data exchange. For our study, described

in chapter 4, isolates collected from an US hospital within a 2-month period were included. In this hospital, VREF are endemic in the ICU and are therefore expected to be genetically highly related. In this study MLVA and PFGE were highly congruent implying that MLVA could replace PFGE for short-term epidemiology purposes.

In chapter 5 analysis of the up- and downstream regions of the *esp* gene revealed that, like in *E. faecalis*, *esp* of *E. faecium* is also contained on a putative pathogenicity island (PAI). Interestingly, except for one other gene, *ara*C, the PAIs of both species are different. Another important difference is the host range of the *E. faecalis* and *E. faecium esp* containing PAIs. Although the *E. faecalis* PAI was found enriched among clinical isolates, it has also been detected in isolates from non-hospitalized persons and in animal isolates. The *E. faecium* PAI has, so far, only been detected in outbreak related and clinical isolates, suggesting a role in nosocomial epidemicity of *E. faecium*. The reasons behind this difference in host range of the *E. faecalis* and *E. faecium* PAI is not known. Analysis of the partially sequenced PAI, including the *esp* gene among several isolates revealed a remarkable sequence heterogeneity with insertions, deletions and mutations, which reflects the enormous plasticity of the *E. faecium* genome.

The *esp* gene encodes the enterococcal surface protein (Esp) thought to be involved in adhesion and biofilm formation. Polymorphisms of Esp may influence tissue tropism or cell surface expression. The exact role of the *E. faecium* Esp is, however, still poorly understood due to lack of an *esp* knockout. Only very recently our research group succeeded in constructing an *esp* knockout, which will enable us to elucidate its role in *E. faecium* pathogenesis.

Several attempts have been made to clone and sequence the up- and downstream regions from the described PAI sequence (chapter 5) to determine the exact size and "ends" of the PAI, but due to mobile elements with high sequence similarity to several copies of similar mobile elements on the *E. faecium* genome, the borders of this PAI have not yet been identified.

MLST typing of bacteria is now a widely used technique for molecular epidemiological studies but can also be used to obtain insight in the population structure and evolution of bacteria. In chapter 6, MLST typing of 411 *E. faecium* isolates, including VREF and vancomycin susceptible *E. faecium* (VSEF) isolates from varies origins, revealed 175 sequence types (STs). Clustering based on the MLST profiles using the eBURST algorithm revealed a population structure, which was characterized by a large cluster with ST22 as predicted primary founder. Within this cluster a smaller complex with ST17 as predicted secondary founder, designated clonal complex 17 (CC17), included most of hospital

outbreak and clinical isolates and was characterized by ampicillin resistance and the presence of the PAI. In a recent review, this population structure, now based on 855 *E. faecium* isolates, was slightly changed and predicted ST26 as primary founder of the large cluster, though grouping of outbreak related and clinical isolates in CC17 remained unchanged (8). In addition to the clustering of clinical relevant isolates, host specific clustering of related STs was observed. For example, human community VREF isolates clustered together with VREF from pigs, suggesting colonization of pig VREF isolates in humans due to consumption of pig meat or direct transmission from animals to humans. Interestingly, human community isolates, ampicillin and vancomycin susceptible, probably representing the commensal population in humans, clustered apart from the main complex. Apparently, the hospital-adapted subpopulation did not directly evolve from the human commensal population.

eBURST predicted ST17 as founder of CC17. So far ST17 is the most globally dispersed *E. faecium* MLST type, identified in nosocomial outbreaks in North and South America, Asia, Europe and Australia and is comprised of PAI positive and negative isolates. Apparently, this clone was very successful in nosocomial spread. At the moment, there are 3 predominant single locus variants (SLVs) of ST17 identified: ST78, ST16 and ST18, respectively (8). ST78 and ST16 have caused documented hospital outbreaks worldwide (2,6,7,9-11,13) and are both PAI positive, while from ST18, although globally dispersed, no documented hospital outbreaks are known and the majority of these isolates are PAI negative (4). These findings contribute to the hypothesis that ST17 by acquisition of the putative pathogenicity island, other adaptive determinants and genetic diversification evolved to a successful clonal complex with increased capability of spread and virulence.

Although we have shown that recombination has played a major role in establishing genetic variation in *E. faecium*, it seems paradoxical that we are still able to detect CC17 as a distinct clonal complex. Therefore, we hypothesize that the emergence of this complex occurred relatively recent and that over time CC17 will fade away, resulting in an *E. faecium* population structure, which resembles a network of a frequently recombining population in which periodically successful clones may dominate.

An unexplained increase of bloodstream infections due to *esp* negative, ampicillin resistant *E. faecium* (AREfm) in our hospital in 2003 prompted the study described in chapter 7. This study showed an ecological replacement of *E. faecalis* by multiresistant CC17 *E. faecium*, illustrated by a decrease in total numbers of invasive enterococcal infections and increase in proportions of invasive AREfm. Furthermore, the ratio *E. faecium*/*E. faecalis* among bloodstream

isolates changed in favor of *E. faecium*, while point prevalence studies revealed high carriage rates of AREfm especially among haematology and nephrology patients. Risk factors for AREfm colonization were diabetes mellitus, three or more admissions in the preceding year and use of β-lactams and quinolones. In the UMCU, haematology patients receive ciprofloxacin prophylaxis during prolonged granulocytopenia, while imipenem is the empirical therapy for granulocytopenic fever. As AREfm are resistant to both groups of antibiotics, selection for AREfm can be explained.

To investigate whether the increase of invasive AREfm was restricted to the UMCU, a nationwide study was initiated (chapter 8). In this study, data on annual numbers of invasive ampicillin resistant enterococci from 26 laboratories in the Netherlands were analyzed and the *E. faecium*/*E. faecalis* ratio among bloodstream isolates provided by 10 laboratories, were determined. The average number of invasive AREfm per hospital increased between 1994 and 2005, and this increase was more pronounced in university hospitals. Proportions of AREfm also increased among bloodstream isolates, again predominantly in university hospitals. MLVA typing of all *E. faecium* isolates revealed spread of 4 types belonging to CC17 in three or more hospitals, including MT-159, which was found in at least 9 hospitals dispersed over the Netherlands in 2006. Furthermore, a sudden increase of *esp* positive isolates, within the most frequent MLVA types, was observed from 2004 on.

The observed increase of AREfm in the Netherlands raises the question, why AREfm replaced *E. faecalis*. An obvious explanation might be selective antibiotic pressure or lapses in infection control. As shown β-lactam antibiotics and quinolone use are risk factors for AREfm colonization. But in the UMCU, quinolone and β-lactam use remained stable over the last 10 years (unpublished data). Only cephalosporin use increased, but it seems unlikely that this has increased selection pressure for AREfm specifically, as all enterococci, including *E. faecalis*, are intrinsically resistant to these agents.

The spread of multiple subclones, all belonging to CC17, without an existing community-reservoir (chapter 7) can only be explained by cross-transmission. Many studies have documented the ability of enterococci to colonize the patients' skin and their prolonged survival in the inanimate environment, both facilitating patient-to-patient spread (3). As a result, standard hygienic measures are frequently insufficient to prevent hospital transmission of enterococci. In the UMCU, a nosocomial outbreak with complex-17 VRE was controlled by combining genotyping and preemptive isolation of patients suspected of carriage (9). In another study, improved environmental cleaning was associated with 31% reductions in VRE-acquisitions rates in ICU-patients (5).

Perspectives

For infection control measures rapid identification of outbreak related *E. faecium* is a prerequisite. The rapid phenotypic test panel can be used for identification of *E. faecium* within 4 hours, while MLVA typing can be used to rapidly determine the genetic relatedness of isolates against low costs. Further studies are necessary to determine whether Raman spectroscopy could be used to identify CC17 *E. faecium* isolates and outbreak related isolates.

For short-term epidemiology like recognition of outbreak related isolates, MLVA typing is a good alternative for PFGE typing. However, in our study (chapter 4), there were several examples of single locus variants of predominant MLVA types with similar PFGE banding patterns, suggesting genetic relatedness of these isolates. Therefore, we propose the following criteria for MLVA typing, as has been done for PFGE (12), for infection control programs in hospitals.

(i) **Indistinguishable**. Isolates are genetically indistinguishable if their MLVA profiles are the same. Though for definitive proof of cross-transmission, epidemiological data like sharing of rooms or overlapping periods of stay in a ward should always be taken into account. To confirm clonal spread of one MLVA type, we recommend to type representative isolates by MLST, as we consider MLST as the new "gold standard" typing technique to determine genetic relatedness among isolates. MLST results can then easily be compared to other worldwide documented outbreaks.

(ii) **Closely related**. Isolates are considered closely related if their MLVA profiles differ only in one of the VNTR loci, so-called single locus variants (SLV). For these isolates, the same considerations as for the indistinguishable isolates to provide proof of cross-transmission are recommended.

(iii) **Possibly related**. Isolates are considered possibly related if their MLVA profiles differ in two of the VNTR loci, so-called double locus variants (DLV). Only in cases of a strong suspicion of cross-transmission the genetic relatedness of these isolates should be confirmed by MLST.

(iv) **Unrelated**. Isolates are considered unrelated if their MLVA profiles differ in more than two of the VNTR loci.

Molecular epidemiological studies in the UMCU indicated horizontal transfer of the *E. faecium* PAI in addition to clonal spread of strains. This was illustrated by the simultaneous finding of isolates belonging to a single clone carrying and lacking this PAI. Vice versa, representative isolates of different clones carried the same PAI subtype. Acquisition of the PAI through horizontal gene transfer may improve the pathogenic properties of clones and increased prevalence, which in itself enlarges the chance of acquiring additional adaptive mechanisms further

enlarging the possibilities for spread. Cumulative acquisition of adaptive mechanisms has been called "genetic capitalism (1)" and reflects the process in which already successful clones only become more successful. Since adaptive genetic elements predominantly represent the accessory genome, addition of these elements to molecular typing schemes will probably gain insights in the mechanisms that contribute to the ecological success of particular clones and may improve the resolution of current typing scheme.

The big challenge resides in what to do with emergence of AREfm in hospitals. There are several options to address this problem; (i) we simply accept the increase of AREfm, while AREfm is already endemic on many wards and implementation of extensive infection control measures, as done during the 2000 VRE outbreak (9) is too expensive or (ii) we improve environmental cleaning as described by Hayden *et al.* (5) to determine whether a similar reduction in AREfm acquisition rates can be obtained as was observed for VRE acquisition rates or (iii) we can try to replace the AREfm by restoring normal microbiota by use of probiotics. In hospitalized patients the residual microbiota is often significantly altered through the detrimental effect of antibiotics. In the UMCU two prospective studies will be performed to determine the last two possibilities. Allowing a further increase in the prevalence of AREfm implies an increased use of vancomycin, linezolid and daptomycin to treat AREfm infections, which might select for either VREF or already described linezolid resistant AREfm and continues the evolutionary rat race, further shaping the selective advantage of multiple antibiotic resistant or virulent bacterial clones.

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Nederlandse Samenvatting

Eenvoudig verhaal

De laatste 20 jaar zijn ziekenhuisuitbraken en infecties veroorzaakt door de - tot dan toe als onschuldig beschouwde - darmbacterie *Enterococcus faecium* sterk toegenomen. Wij hebben studies gedaan naar de genetische achtergrond van deze bacterie en daaruit bleek dat *E. faecium* stammen afkomstig van gezonde vrijwilligers genetisch verschillend waren van *E. faecium* stammen geïsoleerd uit ziekenhuispatiënten. *E. faecium* stammen geïsoleerd uit het ziekenhuis zijn doorgaans resistent voor verschillende antibiotica waaronder ampicilline (tot nu toe eerste keus voor behandeling) en bezitten een specifiek gen dat mogelijk bijdraagt aan betere verspreiding in ziekenhuizen. Uit epidemiologisch onderzoek uitgevoerd in het Universitair Medisch Centrum Utrecht en in 28 Nederlandse ziekenhuizen bleek dat het aantal ernstige infecties veroorzaakt door ampicilline resistente *E. faecium*, vooral bij ernstig zieke patiënten, de laatste 10 jaar opvallend is toegenomen. De toename is met name zichtbaar in universitaire ziekenhuizen. Dit heeft tot gevolg dat andere (meestal duurdere) antibiotica gebruikt moeten worden, die vaak ook meer bijwerkingen hebben en waartegen ook weer resistentie kan ontstaan. Verder onderzoek moet uitwijzen of verspreiding van deze bacteriën voorkomen kan worden of dat risicopatiënten – in de verre toekomst - gevaccineerd zouden kunnen worden tegen deze infecties.

Enterococcus faecium werd jarenlang als een relatief onschuldige, commensale darmbacterie beschouwd, die zo nu en dan infecties kon veroorzaken bij ernstig zieke patiënten. *Enterococcus faecalis* was verantwoordelijk voor 80-90% van de door enterokokken veroorzaakte infecties. Gedurende de laatste 20 jaar is vooral in de VS een sterke toename gezien van ziekenhuisuitbraken en infecties veroorzaakt door glycopeptide resistente enterokokken (zowel *E. faecium* als *E. faecalis*). Deze toename werd voorafgegaan door een toename van ampicilline resistente *E. faecium* (AREfm). Moleculaire typering van een, in eerste instantie, kleine set van vancomycine resistente *E. faecium* (VREF) isolaten wees uit dat ziekenhuis gerelateerde VREF isolaten apart groepeerden van VREF geïsoleerd uit feces van gezonde vrijwilligers. Ziekenhuis gerelateerde VREF werden verder gekenmerkt door resistentie voor ampicilline en chinolonen. Bovendien was de aanwezigheid van het variant *esp* gen sterk geassocieerd met deze uitbraak stammen. In 2001 werd Esp beschreven als een mogelijke virulentie factor van *E. faecalis* waarna werd aangetoond dat het *E. faecalis esp* op een zogenoemd "pathogenicity island" (PAI) ligt.

Dit proefschrift beschrijft de locale, Universitair Medisch Centrum Utrecht (UMCU), landelijke (Nederland) en mondiale moleculaire epidemiologie van *E. faecium*. Verder wordt er een snelle identificatie methode voor enterokokken en een snelle typeringsmethode voor het aantonen van genetische verwantschappen tussen *E. faecium* isolaten beschreven. Tenslotte is een chromosomale regio waar het *E. faecium esp* gen is gelokaliseerd bestudeerd.

Omdat de identificatie van enterokokken controversieel is, hebben we in **hoofdstuk 2** de accuraatheid van verschillende fenotypische testen vergeleken met een moleculaire referentiemethode. Hieruit bleek dat geen van de methoden een 100% betrouwbare identificatie geeft door het voorkomen van atypisch reagerende enterokokken. Het in ons lab ontwikkelde snelle (4 uur) en goedkope identificatieschema bestaande uit een combinatie van al 5 eerder beschreven fenotypische testen, lijkt met een accuraatheid van 92% een goed alternatief voor de duurdere automatische microbiologische systemen en de API. Een andere fenotypische identificatie methode die nog in ontwikkeling is, Raman spectroscopy genaamd, is een veelbelovende methode, maar om de betrouwbaarheid van deze methode goed te kunnen bepalen zal de referentie database eerst uitgebreid moeten worden.

In **hoofdstuk 3** wordt multiple locus variable number tandem repeat analysis (MLVA) als nieuwe snelle typeermethode voor het aantonen van genetische verwantschappen tussen *E. faecium* isolaten beschreven. De methode werd vergeleken met het eerder door ons ontwikkelde multilocus sequence typing (MLST) schema. Deze laatste methode toonde dat ziekenhuis gerelateerde isolaten clusteren. Een nadeel is echter dat MLST duur en tijdrovend is. MLVA bleek in hoge mate congruent aan MLST, waarbij ook met MLVA ziekenhuis gerelateerde isolaten groeperen.

In het kader van infectiepreventie programma's in ziekenhuizen gebruiken veel laboratoria Pulsed Field Gel Electrophoresis (PFGE) als gouden standaard voor het bepalen van kruisbesmetting. PFGE kost echter veel tijd en de typeerresultaten zijn vaak moeilijk te interpreteren, waardoor vergelijking tussen laboratoria problematisch is. Om te onderzoeken of MLVA PFGE als standaard typeermethode in ziekenhuizen zou kunnen vervangen werden in **hoofdstuk 4** beide typeerschema's vergeleken. Hiervoor werden VREF isolaten getypeerd, die gedurende 2 maanden op een ICU waar VREF endemisch voorkomen verzameld waren en waar dus een hoge mate van genetische verwantschap verwacht kon worden. MLVA en PFGE bleken vergelijkbaar te zijn in kosten en het toewijzen van genotypen, maar het discriminerend vermogen van PFGE was iets beter. MLVA is sneller en heeft het grote voordeel dat genotypen vergeleken kunnen worden met een internationale database via Internet. De conclusie van deze studie was dat MLVA een goed alternatief voor PFGE is om verspreiding van isolaten in ziekenhuizen aan te tonen.

In **hoofdstuk 5** zijn de flankerende chromosomale regio's van het *E. faecium esp* gen geanalyseerd, waaruit bleek dat, net als in *E. faecalis*, *esp* in *E. faecium* op een PAI ligt. Op het *esp* gen en één ander gen, *ara*C, na zijn de PAI's van beide species verschillend. Een ander belangrijk verschil tussen het *E. faecalis* en het *E. faecium* PAI is dat het *E. faecalis* PAI, ondanks dat het meer frequent voorkomt in klinische isolaten, ook wordt gevonden in feces isolaten van gezonde vrijwilligers en dieren. Het *E. faecium* PAI is tot nu toe alleen gevonden bij ziekenhuispatiënten. Dit suggereert dat het PAI bijdraagt aan verspreiding van *E. faecium* binnen ziekenhuizen. Uit sequentie analyse van het PAI, inclusief het *esp* gen, van verschillende isolaten bleek een grote mate van heterogeniteit in de vorm van inserties, deleties en mutaties. Dit suggereert een grote mate van genoom plasticiteit in *E. faecium*.

In **hoofdstuk 6** werd de populatie structuur van *E. faecium*, zowel VREF als vancomycine gevoelige *E. faecium* uit verschillende ecologische niches en landen/continenten, opgehelderd met behulp van MLST. Clustering van 175 verschillende genotypen, bepaald in 411 isolaten, met behulp van eBURST toonde één groot cluster van verwante ST's waarbinnen de meerderheid van ziekenhuisuitbraak – en klinische isolaten groepeerden in een subcluster. Omdat eBURST ST17 als vooroudertype van dit subcluster voorspelde, werd dit subcluster clonal complex-17 (CC17) genoemd. Uit het feit dat bijna al deze isolaten gekenmerkt worden door ampicilline resistentie, terwijl het PAI in ongeveer 60% van deze isolaten voorkomt, concludeerden we dat *E. faecium* waarschijnlijk eerst ampicilline resistentie heeft verworven. Dit resulteerde mogelijk in een selectief voordeel in het ziekenhuis, waarna het PAI werd verworven en deze isolaten nog beter in staat waren zich te verspreiden. Verder werd uit deze studie duidelijk dat in *E. faecium* recombinatie een veel belangrijkere rol speelt bij het genereren van genetische diversiteit dan mutaties. De aanwezigheid van CC17 in een panmictische populatie structuur zoals bij *E. faecium* lijkt paradoxaal maar zou verklaard kunnen worden door het feit dat CC17 relatief recent is ontstaan. Het is mogelijk dat in de toekomst CC17 door recombinatie weer zal verdwijnen als apart CC en zal opgaan in een netwerk van frequent recombinerende *E. faecium* populatie, waarin periodiek steeds andere succesvolle clones zullen domineren.

Een opvallende toename van bacteriëmieën veroorzaakt door ampicilline resistente *E. faecium* (AREfm) in het UMC Utrecht in 2003 was de aanleiding voor de studie beschreven in **hoofdstuk 7**. Uit deze studie bleek dat er de laatste 10 jaar een ecologische verschuiving heeft plaats gevonden waarbij er relatief meer infecties worden veroorzaakt door multiresistente CC17 *E. faecium* en minder door ampicilline gevoelige *E. faecalis*. Gedurende de studieperiode was het totaal aantallen invasieve enterokokken infecties afgenomen, maar de proportie invasieve AREfm was toegenomen. Verder nam het aantal *E. faecium* bloedisolaten toe ten koste van het aantal *E. faecalis* isolaten. Uit punt prevalentie studies, uitgevoerd op de afdelingen hematologie en nefrologie, bleek een hoog percentage patiënten drager van AREfm. Risicofactoren voor AREfm kolonisatie waren diabetes mellitus, 3 of meer opnames in het voorafgaande jaar en het gebruik van β-lactam antibiotica en chinolonen.

Om te bepalen of de toename van AREfm UMCU specifiek is, werd een landelijke studie gestart, welke beschreven staat in **hoofdstuk 8**. In deze studie

werd van 26 verspreid over Nederland liggende laboratoria data verkregen over de jaarlijkse aantallen invasieve ampicilline resistente enterokokken. Tevens waren 10 laboratoria bereid om bloed isolaten aan te leveren zodat de *E. faecium*/*E. faecalis* ratio in deze isolaten bepaald kon worden. Uit de analyses bleek dat in de laatste 10 jaar het gemiddelde aantal invasieve ampicilline resistente enterokokken is toegenomen, maar dat deze toename meer uitgesproken is bij universitaire ziekenhuizen. Ook de proportie AREfm in bloedisolaten is toegenomen, ook weer meer uitgesproken bij universitaire ziekenhuizen. Uit MLVA typering van alle *E. faecium* isolaten bleek dat 4 verschillende tot CC17 behorende genotypen in meer dan drie ziekenhuizen zijn voorgekomen. Eén van deze genotypen, MT159, werd in 2006 in minstens 9 verschillende ziekenhuizen gevonden. Verder bleek vanaf 2004 onder de meest frequent voorkomende MLVA typen een toename van *esp* positieve isolaten, wat suggereert dat Esp een rol speelt bij nosocomiale verspreiding van recente *E. faecium* stammen.

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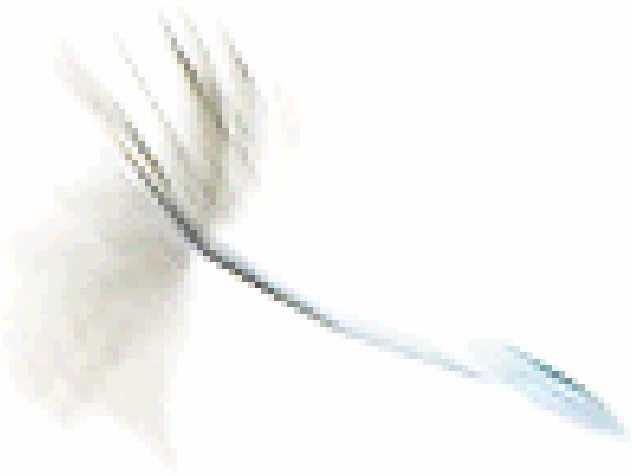

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Curriculum vitae

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De auteur van dit proefschrift werd geboren op 6 augustus 1965 te Woerden. Van 1977 tot 1981 volgde zij haar middelbare schoolopleiding aan de Groen van Prinsterer MAVO te Harderwijk. Na het behalen van haar HAVO diploma aan het Christelijk college Nassau Veluwe te Harderwijk, begon zij in 1983 aan de opleiding Hoger Laboratorium Onderwijs (HLO) medische microbiologie aan het Ir. W.L.Ghijssen instituut te Utrecht. Als onderdeel van deze studie werkte ze mee aan de ontwikkeling van een diagnostische test voor *Treponema pallidum*. Dit onderzoek werd uitgevoerd op de afdeling bacteriologie aan het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven. Direct na het afronden van haar opleiding, begon zij onder leiding van Dr. J. van Embden in september 1987 bij de afdeling bacteriologie van het RIVM aan een nieuw project getiteld Diagnostiek van Humaan pappiloma virus infecties. Van 1990 tot 1992 is zij werkzaam geweest op een project met als doel het tot expressie brengen van heterogene antigenen in *Mycobacterium bovis* BCG. Na haar vaste aanstelling heeft zij tussen 1992 en 1996 een kwaliteitssysteem opgezet volgens Sterlab normen en daarnaast onderzoek gedaan aan *Mycobacterium tuberculosis*. Vanaf januari 1997 deed ze onder leiding van Dr. R. Willems onderzoek naar de transmissie van resistentie genen van dieren naar mensen. Vanaf oktober 2003 werd dit onderzoek verder voortgezet aan het Eijkman Winkler Instituut in het Universitair Medisch Centrum Utrecht en begon zij haar promotieonderzoek onder leiding van Prof. Dr. M. Bonten en Dr. R. Willems. De resultaten van dit onderzoek staan weergegeven in dit proefschrift.