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Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance

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Vancomycin-resistant *Enterococcus faecium* spread dramatically in hospital settings in the USA in the 1990s and reached endemicity at the turn of the century. Similarly, rising prevalence rates are currently observed in several European countries, with prevalence rates of greater than 10% reported in seven of these. On the basis of multilocus sequence typing (MLST), the population structure of *E. faecium* was elucidated and the existence of a distinct high-risk enterococcal clonal complex, designated clonal complex-17 (CC17), which is associated with the majority of hospital outbreaks and clinical infections in five continents, was revealed. This complex is correlated with ampicillin and quinolone resistance and with the presence of a putative pathogenicity island. Preliminary MLST data suggest that similar hospital-adapted complexes might also exist in *E. faecalis*.

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Introduction

Initially considered as a harmless commensal of the gastrointestinal tract, the enterococci have attracted increased public and clinical interest since the first identification of vancomycin-resistant enterococci (VRE) in 1988. In the USA, VRE epidemiology was initially characterized by sporadic, but rapidly increasing monoclonal nosocomial outbreaks, eventually resulting in a polyclonal endemic situation in US hospitals and long-stay health care facilities [1]. Currently, VRE are the third leading cause of bloodstream infections [2]. In contrast, in Europe VRE were initially identified from community sources (i.e. healthy humans and animals). This community-reservoir of VRE presumably resulted from the widespread use of avoparcin (a glycopeptide) as a growth promoter in animal husbandry. After the continent-wide ban of avoparcin in 1997, community prevalence of VRE

declined. Since the early 1990s, sporadic monoclonal nosocomial outbreaks and infections have been reported, mostly from nephrology and hematology wards [3]. However, since the turn of the century, VRE prevalence has been rising in European hospitals. In parallel with this increase in nosocomial VRE infections, the proportion of *Enterococcus faecium* infections has increased relative to those of *Enterococcus faecalis* [4]. These epidemiological events in the USA and Europe demonstrate that the population of hospital-derived enterococci has changed in the last two decades. In this review, recent developments in enterococcal antibiotic resistance and epidemiology are summarized, and how nosocomial enterococcal populations are changing towards predominance of highly specialized enterococcal genetic subpopulations that are capable of surviving and spreading and infecting patients with increasing frequencies in the hospital environment is described.

Antibiotic resistance

Besides being intrinsically resistant to various different classes of antibiotics, enterococci are able to acquire high-level drug resistance through horizontal gene transfer. Examples of resistance genes that have been acquired by enterococci include those that confer resistance to aminoglycoside, macrolide, streptogramin and chloramphenicol, with resistance to vancomycin being the most clinically relevant. *E. faecium* tends to accumulate ampicillin and vancomycin resistance more frequently than does *E. faecalis* [5].

Resistance genes are not only transferred among enterococci, but also between enterococci and more pathogenic bacteria. The transmission of the vancomycin-resistant transposon from VRE to methicillin-resistant *Staphylococcus aureus* (MRSA), creating a vancomycin-methicillin-resistant *S. aureus* (VRSA) strain in a hospitalized patient represents a dramatic example [6]. To date, five cases of VRSA have been reported from the USA: three cases in Michigan, one in Pennsylvania and one in New York. Importantly, there was no epidemiological linkage between these patients, and each case is considered a separate occasion of *de novo* resistance-development.

Treatment options for VRE-infections are limited as these bacteria are frequently resistant against most of the available antimicrobial agents [7]. Several newer antibiotics are now available, including quinupristin-dalfopristin, linezolid and daptomycin [8]; yet, for each of these recently introduced antibiotics, resistance has

already been reported [9,10^{*},11,12,13^{*},14,15]. Importantly, for quinupristin-dalfopristin, to which *E. faecalis* is intrinsically resistant, a community-reservoir for resistance already exists in the USA and Europe, presumably because of widespread use of its analogue, virginiamycin, as growth promoter [16]. Tigecycline is a new anti-enterococcal drug with promising activity for which FDA approval was obtained mid-2005 [17].

Epidemiology

At the turn of the century, VRE colonization was considered endemic in most US hospitals and the prevalence of VRE among enterococcal bloodstream infections in intensive care units (ICUs) appears to have stabilized at ~28% since then [2]. Yet, several reports suggest that VRE has recently become more prevalent in non-ICU populations, such as patients receiving hemodialysis, and pediatric patients in hematology-oncology departments [18]. The stabilization of the VRE rate at 28% closely approximates the percent of enterococcal infections caused by *E. faecium*, indicating that vancomycin-resistance has almost completely penetrated *E. faecium* populations in the clinical setting.

In Europe, nosocomial outbreaks and clinical infections with VRE seem to have emerged since the turn of the century. In the most recent European Antimicrobial Resistance Surveillance System (EARSS) report (2005, to be published) prevalence rates of VRE among enterococcal bloodstream infections were reported to be greater than 10% in seven European countries, with five countries reporting prevalence rates of greater than 20% (Israel, 45.7%; Cyprus, 40%; Portugal, 33.7%; Ireland, 30.9%; Greece, 29.1%; Czech Republic, 13.7%; and Germany, 13%; data retrieved on May 23, 2006 from the EARSS, available at <http://www.earss.rivm.nl>). Even in countries with traditionally low antimicrobial resistance levels, the epidemiology of multi-resistant enterococci appears to be changing. In Norwegian, Swedish and Finnish hospitals, endemicity of ampicillin-resistant *E. faecium* was established [19–21], with documented events of acquisition of vancomycin resistance [20,22].

Nosocomial VRE outbreaks are also reported with increasing frequency from Asia and Australia [23–26]. In contrast to the USA and Europe where *vanA* is the predominant resistance mechanism among VRE, resistance through *vanB* predominates in *E. faecium* from Australia and Singapore [23,25]. Vancomycin resistance rates also rose from 0% to 5% in Latin-American enterococcal bloodstream infections [27].

Different genotyping methods have been used to study enterococcal epidemiology, such as pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) analysis and multilocus sequence typing (MLST). MLST is based on variations in (silent)

mutations in short sequences from seven housekeeping genes, it is highly reproducible, provides unambiguous genotyping results and is now generally considered the gold standard for studying *E. faecium* genetic relationships [28].

Recently, two different MLST schemes have been developed for studying the epidemiology of *E. faecalis*. With the first scheme, which includes six housekeeping genes and three virulence genes, 21 *E. faecalis* strains that were isolated mainly from US hospitals were analyzed [29^{*}]. A second MLST scheme, based on seven housekeeping genes, was used to study the genetic-relatedness of 110 strains from different sources and geographic regions, although with an over-representation of Spanish isolates [30^{*}]. This second scheme appeared to have similar discriminatory power to that of PFGE-typing.

In contrast to the overt advantages of MLST — being reproducible, portable, highly discriminative and unambiguous — performing MLST is time-consuming and expensive. As an alternative, multiple-locus variable-number tandem repeat analysis (MLVA) has been developed [31,32]. Using the Simpson's index of diversity, MLVA of *E. faecium* was equally discriminative; MLST, AFLP and clustering of isolates upon MLVA-profiles yielded comparable genogrouping. Thus, MLVA seems a quick and cheap alternative to identify hospital outbreaks and to study the global epidemiology of *E. faecium* and *E. faecalis*.

Population structure and genetic evolution of *E. faecium*

In addition to epidemiological investigations, molecular typing data can also be used to examine patterns of evolutionary descent and obtain knowledge on the population structure of bacterial pathogens. A first insight into the population structure of *E. faecium* was gained by the AFLP analysis of strains from different human and animal origins. AFLP revealed the existence of different host-specific genetic lineages, with isolates recovered from veal calves, poultry and pigs clustering in distinct genetic branches [33]. Vancomycin-resistant *E. faecium* (VREF) isolated from human volunteers clustered together with the pig isolates, whereas VREF isolates associated with documented nosocomial outbreaks and infections clustered in a distinct branch, initially designated 'lineage C1' [33]. Ribotyping, as well as a first analysis of 139 isolates by MLST, confirmed the existence of host-specific lineages and a distinct genetic subpopulation representing clinical and hospital outbreak isolates [28,34]. A more extensive MLST-analysis of 411 *E. faecium* isolates (179 of which are VRE) from different human and animal sources and different continents explored the evolutionary origin of epidemic isolates by discerning patterns of microevolution within the C1 lineage and determined the population structure of *E. faecium* [35^{••}]. *E. faecium* ST17

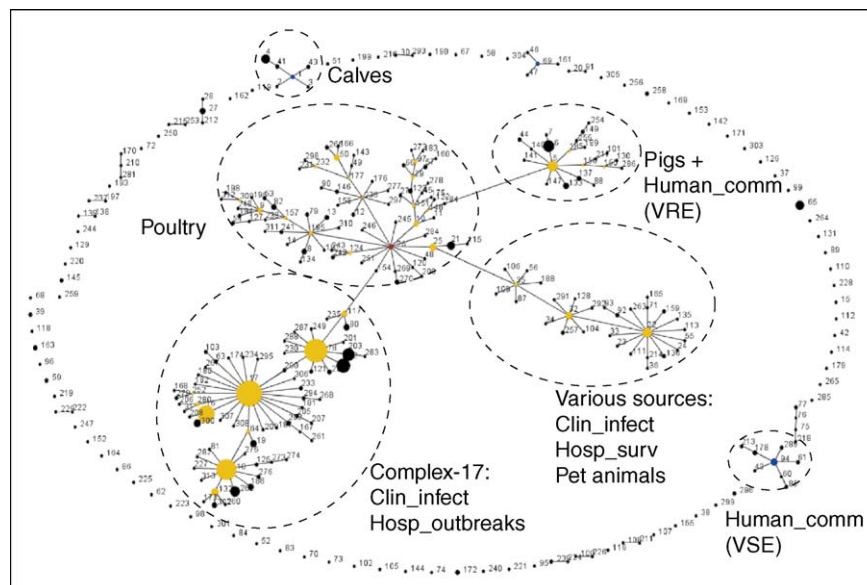
is the predicted founder of the C1 genetic lineage, which apparently adapted to and exploited the hospital ecological niche. Through genetic diversification, including mutations, but primarily recombination, this primary ancestor evolved into a meroclone of highly related genotypes, which has spread globally (USA, Europe, Asia, Australia, Africa and South-America). This genetic subset, renamed clonal complex-17 (CC17), seems to be responsible for the worldwide emergence of nosocomial VRE, as most of the infection-related and outbreak strains belong to this complex. Clonal spread of this high-risk enterococcal clonal complex has been reported from hospitals in the Netherlands [36,37], the USA, UK and Australia [35^{••}], Italy [9,38,39,40[•]], Germany [13,41], Sweden [42], Korea [24] and Singapore [25]. As yet, only four of 38 nosocomial VRE-outbreaks included in the aforementioned MLST-analysis (one from Brazil, one from the USA, one from Greece and one from the Netherlands) did not belong to CC17 (Figure 1) [43,44].

Even in countries with few documented VRE outbreaks, such as Spain and Norway, CC17 has colonized and infected hospitalized patients [45[•],46]. Here, CC17 isolates were vancomycin-susceptible but uniformly resistant to ampicillin, suggesting that ampicillin resistance has been one of the adaptive mechanisms acquired by CC17. In addition to being ampicillin-resistant, CC17 isolates are also characterized by higher levels of quinolone

resistance [21,47] and the presence, at least in the majority of CC17 isolates, of a putative pathogenicity island [48] carrying the *esp* gene [35^{••},49,50]. Furthermore, *hyl*, another recently discovered putative virulence gene encoding a hyaluronidase [51], was enriched in CC17 isolates [13[•],52]. All of these recent findings suggest that hospital-adaptation of CC17 has been a multi-step process involving the sequential acquisition of adaptive mechanisms that provide CC17 with selective advantages, thus enabling the acquisition of yet more adaptations. This has eventually resulted in a genetic subpopulation that is highly specialized for survival and spread in hospitals; this process is called 'genetic capitalism' [53] or the 'Mathew effect' (Figure 2). Our observation that recombination has played a major role in establishing genetic variation in *E. faecium* is in line with this hypothesis, because it is most probable that acquisition and incorporation of adaptive mechanisms has occurred through horizontal gene transfer and recombination [35^{••}].

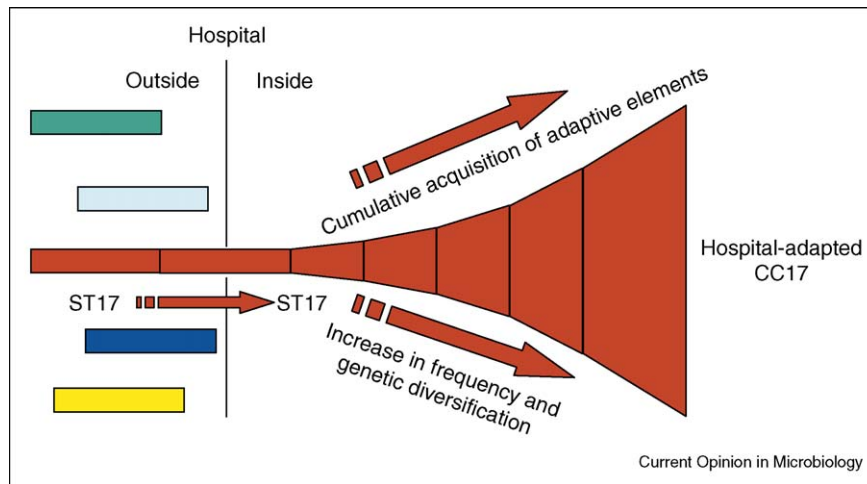
The stronger association between CC17 and ampicillin resistance, compared to that between *esp* and *hyl* and CC17, suggests that ampicillin resistance was among the first adaptive mechanisms acquired by CC17 *E. faecium* [35^{••}]. If this is true, the rapid emergence of ampicillin resistance in the USA in the early 1980s [54,55] might, in retrospect, have represented one of the first steps of hospital-adaptation by CC17.

Figure 1



A population snapshot of 855 *E. faecium* isolates on the basis of MLST allelic profiles using the eBURST algorithm [57]. This snapshot shows all clonal complexes, singletons and patterns of evolutionary descent. The relative size of the circles indicates their prevalence in the MLST database (<http://www.mlst.net/>). Numbers correspond to the sequence types (STs), and lines connect single locus variants: STs that differ in only one of the seven housekeeping genes. CC17, the major subpopulation representing hospital outbreaks and clinical infections, is indicated, as well as the source of other major subgroups. Annotations: Clin_infect, isolates from clinical sites (mainly blood) from hospitalized patients; Human_comm, faeces isolates from human volunteers not connected to hospitals; Hosp_outbreak, isolates from hospital outbreaks; Hosp_surv, faeces isolates from hospitalized patients without an enterococcal infection and not associated with an enterococcal hospital outbreak; VSE, vancomycin-susceptible enterococci.

Figure 2



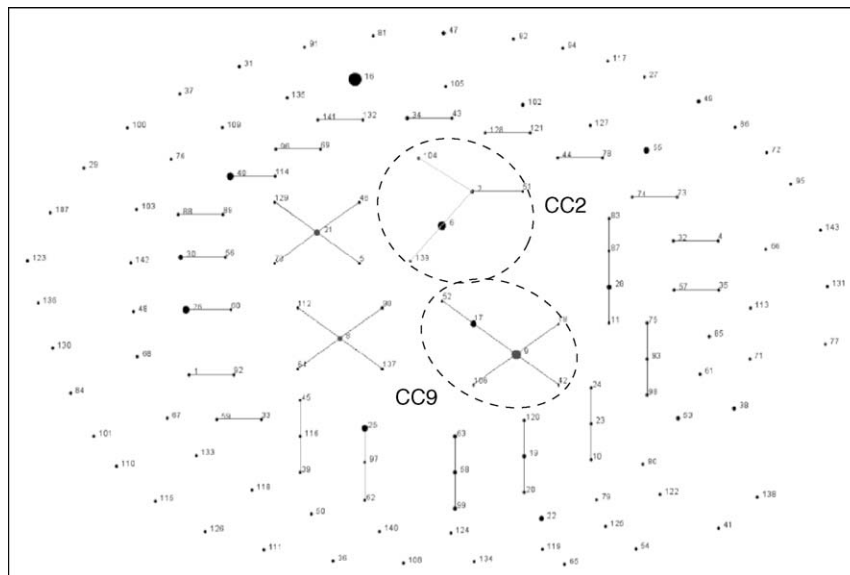
Hypothetical scheme describing the different events that have led to the ecological abundance of CC17 in hospitals. Through acquisition of multiple adaptive mechanisms, including the sequential acquisition of resistance genes and putative virulence genes, and facilitated by events of horizontal gene transfer and recombination ST17 (the presumed founder of CC17) gains a selective advantage and is able to exploit the hospital environment. This is followed by an increase in frequency facilitating further adaptive possibilities and genetic diversification resulting in a cluster of closely-related genotypes (CC17), which are all descended from the founding genotype, ST17, and well adapted to the hospital environment. This process has been called genetic capitalism (the rich tend to become richer) or the Matthew effect ("For unto every one that hath shall be given, and he shall have abundance: but from him that hath not shall be taken away even that which he hath." (Matthew XXV:29, The Bible [King James Version]).

Population structure of *E. faecalis*

The population structure of *E. faecalis* has not yet been studied as extensively as that of *E. faecium*. MLST-analysis of 21 *E. faecalis* isolates suggested the existence of two important clonal complexes, designated BVE for Bla⁺ Van^r

endocarditis and ACB for Argentina-Connecticut-Bla⁺ [29*]. The BVE clonal complex, which is comprised of 11 *E. faecalis* strains that are resistant to vancomycin and have high-level resistance to gentamicin, produced a β -lactamase and carried the previously described

Figure 3



Population snapshot of 229 *E. faecalis* isolates on the basis of MLST allelic profiles using the eBURST algorithm [57]. This snapshot shows all clonal complexes, singletons and patterns of evolutionary descent. The relative size of the circles indicates their prevalence in the MLST database. Numbers correspond to the STs, black lines connect single locus variants and blue lines connect double locus variants, STs that differ in two of the seven housekeeping genes. The high-risk enterococcal clonal complexes CC2 and CC9, exclusively containing hospital related isolates, are indicated.

E. faecalis pathogenicity island [56]. Isolates that belong to this clonal complex have caused hospital outbreaks and life-threatening infections. The ACB clonal complex (containing two *E. faecalis* strains) also consisted of β -lactamase-producing isolates that had been recovered from patients in the USA and Argentina. Using a different MLST scheme, Ruiz-Garbajosa *et al.* [30] typed 110 isolates originating from humans and animals from 10 countries in Europe, Asia and America. Despite random dispersion of human clinical isolates, surveillance isolates and animal isolates, two complexes (CC2 and CC9) that exclusively contained hospital-derived isolates were identified (Figure 3). As is the case for *E. faecium*, this suggests that an adaptation to the hospital environment has occurred in *E. faecalis* as well. With this MLST-scheme, strains from the BVA clonal complex clustered in CC2, whereas the ACB clone clustered with CC9. On the basis of the few data available, the *E. faecalis* population structure appears to be epidemic, with extensive recombination and without host specificity. The frequency of recombination among *E. faecalis* seems to be higher than among *E. faecium*. In line with the findings in *E. faecium*, CC2 and CC9 might be designated high-risk enterococcal clonal complexes of *E. faecalis*.

Conclusions

Recent molecular epidemiological studies have identified CC17, a globally dispersed clonal lineage of *E. faecium*, characterized by resistance to ampicillin and quinolones. After acquisition of vancomycin-resistance, which is by no means associated with CC17, it was apparent this *E. faecium* population had become emerging nosocomial pathogens, first in the USA and now in Europe and other continents as well. CC17 probably became highly successful in the hospital environment through successive acquisition of multiple adaptive mechanisms, such as antibiotic resistance, amongst others. This process of genetic capitalism might have been favored by the high recombination potential of *E. faecium*. Emergence of epidemic clones has also been observed in other nosocomial pathogens (i.e. MRSA and methicillin-resistant *Pseudomonas aeruginosa*), but evolution, possibly relatively recently, of a single genetic complex seems unique to *E. faecium*. As ampicillin resistance is a very specific genetic marker of CC17, increasing infection rates of ampicillin-resistant enterococci (ARE) represent the first sign of the emergence of CC17 in hospitals. In countries with low levels of VRE, emergence of ARE might set the stage for nosocomial VRE-outbreaks, especially where vancomycin-resistance genes are abundantly present in the extramural animal and human population. Nosocomial spread of CC17 ARE has recently been reported in Norway [46], Spain [45*] and Korea [52].

Although *E. faecalis* probably still causes more infections than *E. faecium*, the former appears to be less efficient in accumulating resistance. The population structure of *E.*

faecalis seems epidemic, like that of *E. faecium*, with two clonal complexes consisting solely of clinical and hospital outbreak isolates. Nevertheless, clinical and outbreak strains are in general more dispersed over different genetic backgrounds than is the case in *E. faecium*. Interestingly, recombination frequencies seem to be higher in *E. faecalis*. The reason why *E. faecalis* has been less successful in acquiring resistance mechanisms has yet to be determined.

These recent studies illustrate the changing epidemiology within enterococcal populations; High-risk clonal complexes, characterized by high-level multiple antibiotic-resistance traits, high levels of recombination and possibly more virulence and transmissibility, seem to replace the more heterogeneous and antibiotic-susceptible populations. This directly implies less therapeutic options for critically ill patients that are infected with these opportunistic pathogens. Moreover, because of this emergence, a nosocomial reservoir of resistance genes, with a proven potential for horizontal gene transfer to more pathogenic bacterial species, is being established.

Acknowledgements

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