Enterococcus faecium: from fundamental insights to practical recommendations for infection control and microbiological diagnostics

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SUMMARY

Enterococcus faecium has rapidly become a successful nosocomial pathogen. Early in its evolution *E. faecium* already possessed traits such as high tenacity, resistance to antibiotics and environmental stresses which made it capable to survive in a hospital environment. The adaptation to the human gastrointestinal (GI) tract was already developed in the very beginning and became even more sophisticated during the urbanization of humans. The wide use of antibiotics was another driver in the further evolution of *E. faecium*. From that time on the genetic capitalism of this organism became very clear. The genome of *E. faecium* seems so flexible that it can easily adapt in response to environmental changes, including the hospital environment. Through the continuous acquisitions and refinements of successful adaptive traits, *E. faecium* belonging to the hospital lineages have become highly proficient nosocomial pathogens.

We aimed to incorporate the evolutionary insights into practical infection control guidelines, in order to reduce the spread of successful lineages of *E. faecium*. If we aim to prevent vancomycin resistant *E. faecium* (VREfm) infections, reducing VREfm carriage and spread is essential as well as challenging. Important examples of infection control measures are: intensified cleaning procedures, antibiotic stewardship, rapid and adequate screening of VREfm carriage and rapid and accurate typing in outbreak cases. This review is intended to provide a guideline on infection control practice, in view of the biological properties of this microorganism. Finally, innovations in the fields of diagnostics, treatment, and eradication is necessary to tackle the ongoing success of *E. faecium*.

INTRODUCTION

Recent examination of the evolutionary history of enterococci revealed that the genus Enterococcus originated 425-500 million years ago from the ancestor *Vagococcus*. Vagococci resided in marine environments and were able to colonize ecologies with high levels of bile, a characteristic feature also in enterococci. Life on land exposed the bacteria to dry conditions and starvation. Compared to their ancestor, enterococci developed a thickened cell wall and coping mechanisms to environmental stresses. Due to these evolutionary changes, enterococci have become highly tenacious microorganisms [1].

Enterococci were first discovered in the human fecal flora in 1899. Until 1984, they were part of the genus Streptococci [2]. *Streptococcus faecalis* was first described in 1906, when the microorganism was isolated from a patient with endocarditis. *Streptococcus faecium* was first detected in 1919. Later on, streptococci belonging to serogroup D were divided into two groups. The division was made based upon biochemical differences and differences from nucleic acid studies (DNA-rRNA homology studies and 16SrRNA) [3]. *Streptococcus faecalis* and *Streptococcus faecium* were placed in the enterococcus group, to which nowadays more than 50 species are belonging [4].

In the seventies and eighties enterococci emerged as a leading cause of hospital associated (HA) infections [5]. Among the enterococci, *E. faecalis* and *E. faecium* are the main causative agents of infection in humans. In the past two decades, especially *E. faecium* has rapidly evolved as a nosocomial pathogen worldwide. Not only has *E. faecium* successfully adapted to the conditions to survive in the nosocomial setting, but also has this species commonly acquired resistance against glycopeptides located on mobile genetic elements (MGEs) carrying *vanA* or *vanB* genes [6].

As described above, early prehistoric conditions in the times of early speciation of bacteria already made that enterococci have become a tenacious microorganism by nature. In this review, we will further focus on the successful evolutionary events of *E. faecium*. Throughout this review we will describe several successful traits and conditions that have had a high impact on the shaping of *E. faecium* as a successful nosocomial pathogen. Secondly, we describe the historical rise of *E. faecium* infections in hospitals worldwide, followed by the subsequent emergence and epidemiological background of vancomycin resistant *E. faecium* (VREfm). Finally, we review the influence of the conditions in the modern hospital settings, in which *E. faecium* has emerged as an important pathogen over the past 20 years. We aim to translate the insights, based on evolutionary research, of how *E. faecium* has become such a successful nosocomial pathogen, to practical infection control guidelines to withstand the spread of the HA lineages of *E faecium*.

THE EVOLUTION OF *ENTEROCOCCUS FAECIUM* IN THE ANTIBIOTIC ERA: INCREASE IN RESISTANCE AND VIRULENCE

Population genetics and genomics showed that the current two different lifestyles of *E. faecium*; commensals of the gastrointestinal (GI) tract and an opportunistic pathogen of critically ill patients, are represented by distinct subpopulations. The presence of these distinct subpopulations was already recognized two decades ago using a fingerprint-based typing method, amplified fragment length polymorphism [7]. Later sequence-based methods such as multi-locus sequence typing (MLST) and whole genome sequencing (WGS) confirmed and further described these distinct *E. faecium* subpopulations [8-10]. Currently, the animal and hospital lineages are designated as clade A, the human commensal lineages as clade B [11].

The divergence of the human commensal *E. faecium* lineage from the animal and hospital lineages is predicted to have occurred about 3000 years ago [12]. Around that time period, humans started to live more and closer together in cities. In addition, increased domestication and the feeding of animals may have had influence on the diet of these animals [12]. The divergence of these two clades went together with replacement of redundant metabolic pathways. Specifically, differences in carbohydrate utilization marks the differences between the two subclades of *E. faecium*. Human commensal strains can very well metabolize carbon derived from dietary sources, whereas animal and HA strains utilize host secretions and cell surface modifications as carbohydrate sources [13].

The currently successful hospital lineages belong to a subclade of clade A, A1, previously designed as clonal complex 17 (CC-17) [14]. Clade A further contains non-clade A1 strains, which forms a number of subclades containing animal related isolates and early clinical *E. faecium* isolates [15]. The divergence of clade A1 from the other clades in clade A coincided with the introduction of antibiotics in clinical care.

Genetic capitalism of the hospital associated *Enterococcus faecium*

The evolution of *E. faecium* is characterized by specialization in order to adapt and survive in a wide range of ecological niches, representing a wide range of selective pressures. Isolates belonging to the HA subpopulation are characterized by ampicillin resistance, pathogenicity islands and are associated with hospital outbreaks [10]. In addition, genome wide studies have shown that these HA isolates acquired a number of traits making them successful in the hospital environment. These strains contain more antibiotic resistance genes and virulence genes enhancing biofilm formation and colonization [16]. Gene flux and capture of adaptive

traits, the result of gene acquisition and gene loss in *E. faecium,* is facilitated by plasmid transfer and through homologous recombination where insertion sequence (IS) elements may provide homology at specific sites [9]. Furthermore, *IS* elements enable a high frequency of rearrangements leading to new genomic configurations further facilitating adaptation under strong selective conditions like the hospital environment. Bayesian analysis of the population structure of *E. faecium* suggested that once particular clones or lineages were adapted to the hospital environment, recombination declines [14]. The continuous refinement of genomic configuration, characterized by the flux and integration of successful adaptive traits, will result in a selective advantage and clonal expansion, which in itself, increases the probability of acquiring additional adaptive traits. This process of cumulative acquisition of adaptive traits following clonal expansion has been coined genetic capitalism [17] (Figure 1).

Increase of *Enterococcus faecium* infections in hospitals

Around 2000, infections due to ampicillin resistant *E. faecium* (AREfm) started to raise in Europe, replacing *E. faecalis* infections [18]. In fact, the European Antimicrobial Resistance Surveillance System (EARSS) data of 2002-2008 showed the largest increase (on average annually 19.3%) in the number of positive *E. faecium* blood cultures compared to the increase of other pathogens as *E. coli, S. aureus, S. pneumoniae* and *E. faecalis* [19]. This emergence of *E. faecium* BSIs was also observed in the University Medical Center Groningen (UMCG, The Netherlands). Figure 2 shows the ratio of positive blood cultures with *E. faecalis* and *E. faecium* in individual patients during 1998-2017. While the incidence of *E. faecalis* BSIs remained rather constant, the *E. faecium* to *E. faecalis* ratio changed approximately from 0.1 in 1998 to 1.6 in 2017. As described above, these AREfm genotypically belonged to what was then named CC-17 [20] and which is now known as the HA clade A1. Also, individual hospitals in Europe, including Ireland, Spain, Poland, Denmark and Switzerland have reported the increase of *E. faecium* bloodstream infections (BSI) to be associated with successful CC-17 clones [21-25]. Furthermore, countries outside Europe observed increasing infections with *E. faecium.* The USA observed an increase in *E. faecium* BSI since 2002, with a peak in 2010 with a prevalence of 5.4% and fortunately, since then decreasing [26]. A recent overview of the contribution of antimicrobial-resistant pathogens causing HA infections in the US during 2011-2014, shows that the overall contribution of *E. faecium* was 3.7% [27]. The contribution was highest in catheter-associated urinary tract infections. Also the Australian Enterococcal Sepsis Outcome Program (AESOP) 2014 reported that a large proportion (39.9%) of enterococcal bacteremia were caused by *E. faecium* [28].

Figure 1: Model of evolution of *E. faecium* marked by the cumulative acquisition of adaptive traits following clonal expansion. Adapted from Fernando Baquero. From pieces to patterns: evolutionary engineering in bacterial pathogens. Nature Reviews in Microbiology 2004

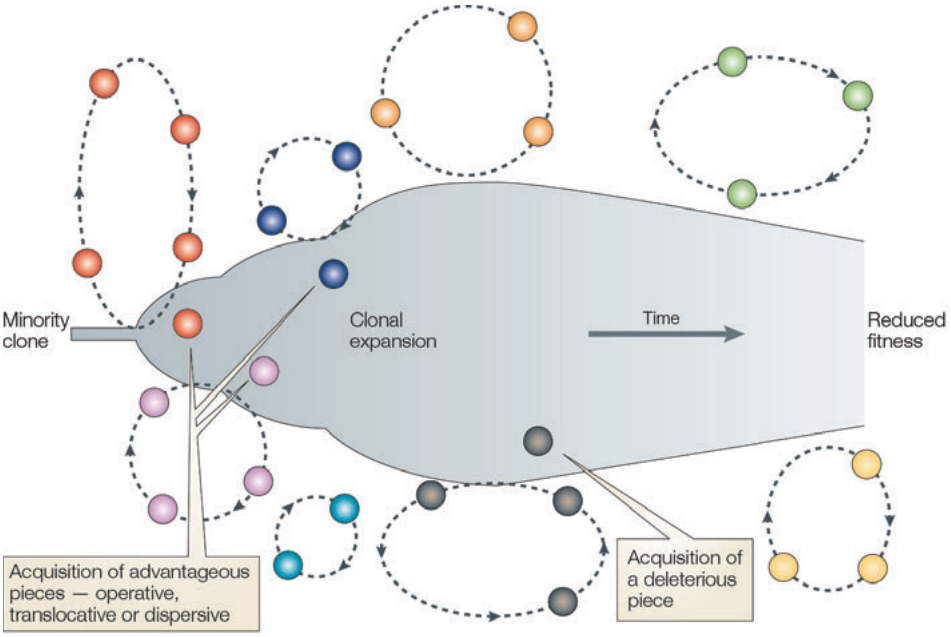
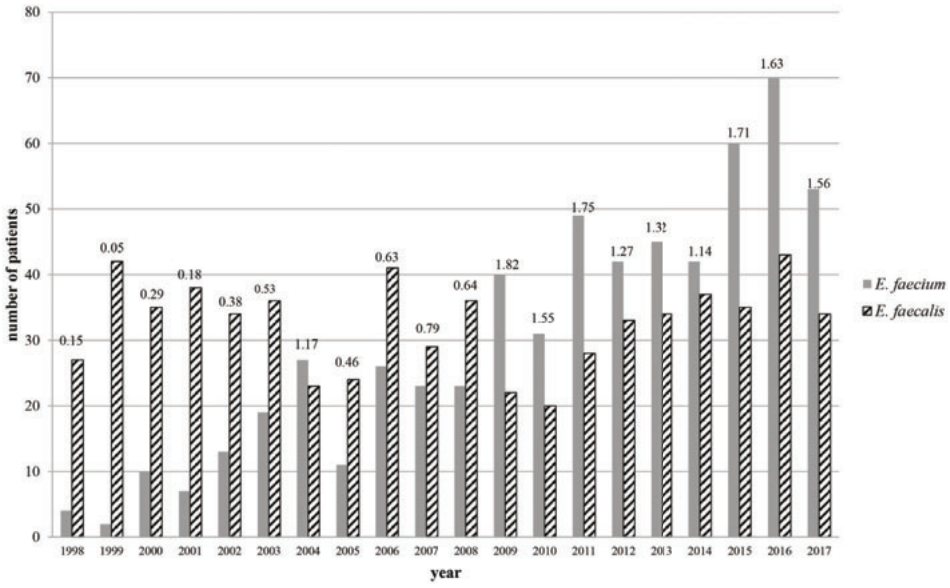


Figure 2: Number of patients with blood cultures with *E. faecium* and *E. faecalis* in individual patients and the *E. faecalis/E. faecium* ratio during 1998-2017 in the University Medical Center Groningen. The *E. faecium* to *E. faecalis* ratio changed approximately from 0.1 in 1998 to 1.6 in 2017.



Enterococcus faecium colonization and colonization resistance

BSIs with *E. faecium* mainly occur in hospitals in patients with underlying disease (oncology-hematology patients) and are associated with prior antibiotic use and prior *E. faecium* colonization [21, 29-31]. Prior (heavily) colonization with *E. faecium* is associated with the subsequent development of a BSI with *E. faecium* [29-31]. When enterococci proliferate to a high density in the GI tract, antibiotic resistant strains can cause disease by translocating to deeper tissues and to the bloodstream [32]. Treatment with antibiotics such as metronidazole inhibiting anaerobic bacteria, can lead to a profound proliferation of VRE in the GI tract and can subsequently result in BSI [33, 34]. Both direct and indirect immune responses are involved in the colonization resistance of intestinal pathogens. Especially anaerobic intestinal flora seem to be protective against overgrowth by enterococci. Commensal bacteria such as *Bacteroides thetaiotaomicron* play an important role in impairing the colonization of VRE. These bacteria enhances the expression of the peptidoglycan-binding C-type lectin regenerating islet-derived protein III (REGIII), an antimicrobial peptide that targets and kills Gram-positive bacteria. Other microbial products such as lipopolysaccharide (LPS) and flagellin stimulate Toll-like receptor (TLR) 4+ stromal cells and TLR5+CD103+ dendritic cells (DCs) also enhance the epithelial expression of REGIII [35]. Thus, antibiotic mediated depletion of commensal bacteria associated with a decrease of REGIII can lead to enterococci outgrowth in the GI tract. Moreover, some anaerobic bacteria can even clear VRE colonization. A study of Caballero et al. demonstrated that a combination of four anaerobic bacteria provides colonization resistance to VRE *in vivo*, and that especially *Blautia producta* is an important contributor to VRE inhibition [36]. In another study, *Barnesiella* was found to cure patients from VRE colonization and subsequent bloodstream infection with VRE [33, 37].

The rise of vancomycin resistant enterococci (VRE)

The acquisition of resistance against glycopeptides is an important landmark in the evolution of enterococci towards a highly resistant microorganism. (Van-A-type) VRE was first reported in 1988 in France and the United Kingdom [38, 39]. Nowadays most VRE outbreaks are due to HA-VSEfm that acquired the *vanA* or *vanB* gene [40, 41].

VanA-type VRE dominated the epidemiology of VRE in the United States (US) and Europe [42]. In the US VRE already emerged in 1990 while still being rare in hospitals in Europe. Like in Europe, the emergence of AREfm in the 1980s [43] preceded the emergence of VREfm in the 1990s in the US hospitals [44]. Data from the Centers for Disease Control and Prevention (CDC) about HA infections caused by antibiotic resistant bacteria from 2011-2014, show a high but decreasing prevalence of VREfm in the US, from 80.5% in 2011 to 75.6% in 2014 [45].

In Europe, hospital infections with AREfm started to increase from 2000, followed by an increase in VRE [41] similar of what happened in the US 20 years before (Figure 3). However, the situation in Europe differed from that in US. In contrast to the US, Europe did have a large reservoir of VRE in the community in the 1990s, yet without suitable HA AREfm populations in hospitals to take up the *van* genes and become HA VREfm. This large reservoir of VRE in the community and farm animals was linked to the avoparcin use in husbandry [46, 47]. Avoparcin was not used in the US and a community reservoir of VRE was therefore absent [48]. In the US, the rise in VRE was probably due to the extensive use of antibiotics [49] in humans along with failures in infection prevention measures leading to cross transmissions [50]. Avoparcin a glycopeptide antibiotic like vancomycin, has been used since 1970 as a growth promotor in the agricultural sector in several European countries. Its use was associated with high numbers of *vanA* VRE in meat and animals [51]. Because of the potential risk of transmission of VRE or *van* genes from the community into the hospitals, the use of avoparcin was banned in European countries in 1997. As a result, VRE in farm animals declined rapidly. However, persistence of vancomycin resistance in *E. faecium* in broilers and poultry farms has been reported in several countries [52, 53]. It is not known to which extend these mobile genetic elements (MGEs) such as (*vanA*) transposons are still a potential reservoir for HA VREfm [54, 55].

Data from the European Centre for Disease Prevention and Control (ECDC) for 2016 show considerably variable surveillance data for VREfm between the European countries [56]. For example, the proportion of VREfm is <1% in Sweden, Finland, the Netherlands and France, while Ireland reports the highest proportion of 44.1% (Figure 4). Remarkable are the rapid increasing trends in especially Eastern European countries like Romania, Latvia, Lithuania, Poland, Hungary, Slovakia, Croatia, Cyprus and Bulgaria (Figure 5). The ECDC surveillance Atlas on Antimicrobial resistance reports VREfm proportion rates for these countries in 2016 as follows: Romania 39%, Latvia 28.6%, Lithuania 21.3%, Poland 26.2%, Hungary 22.4%, Slovakia 26.4%, Croatia 22.1%, Cyprus 46.3% and Bulgaria 18.2%. Little is known about which lineages and *van*-types are involved in the significant increase of VREfm in these countries. A prospective study from Bosnia and Herzegovina and Croatia from 2013, showed that 80% (28/35) of their randomly tested *E. faecium* isolates were vancomycin resistant, of which 71.4% harbored the *vanB* gene and 26.6% the *vanA* gene [57]. A recent study from Poland reported an increasing prevalence of VREfm with a changing epidemiology towards *vanB* VREfm [58]. Importantly, besides in the aforementioned countries, *vanB* VRE do seems to emerge in several European countries since 2005, amongst others in Spain, Greece,

Sweden, Germany and France [59-65]. Hospitals in Sweden had a low prevalence of VRE and incidentally *vanB* VRE was seen. In 2007, outbreaks in three Swedish hospitals occurred and further clonal dissemination with *vanB* VRE was seen [62, 63]. In Germany, *vanB* VRE seems to emerge since 2010, and was typically associated with lineage ST192 [64]. Recently, Germany have noted a higher number of *vanB* VRE compared to *vanA* VRE in 2016 [66]. Also, in France the proportion of *vanB* VRE increased rapidly from 2.2% to 39.3% between 2006 and 2008 [65].

In the Netherlands, the proportion of *vanB* VRE is also quite significant. Of the 706 VRE strains that were analyzed between May 2012 and March 2016 from 42 Dutch hospitals, 363 carried the *vanA* gene, 340 the *vanB* gene, four both the *vanA* and *vanB* gene and two carried the *vanD* gene [67]. The increase of *vanB* VRE is not yet fully understood. It could be linked to the expansion of specific lineages which might be more successful in incorporating *vanB* elements into their genome. For example, ST192, ST203 and ST117 seem to be responsible for the majority of *vanB* VRE in Germany, Australia and Sweden (63, 64, 68). In contrast, these STs were responsible for causing *vanA* VRE outbreaks in Denmark [69].

Figure 3: Course of events in the epidemiology of AREfm and VREfm and the differences between the USA and Europe. HGT= horizontal gene transfer. Blue. Hospital Clade A1-VSEfm (AREfm); Red. Hospital-Clade A1 VREfm.

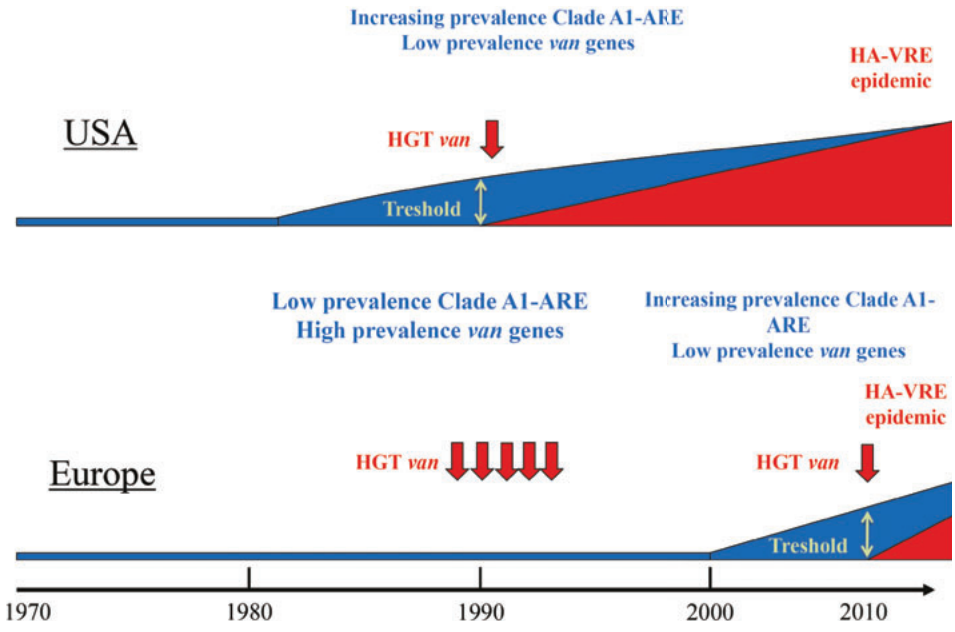
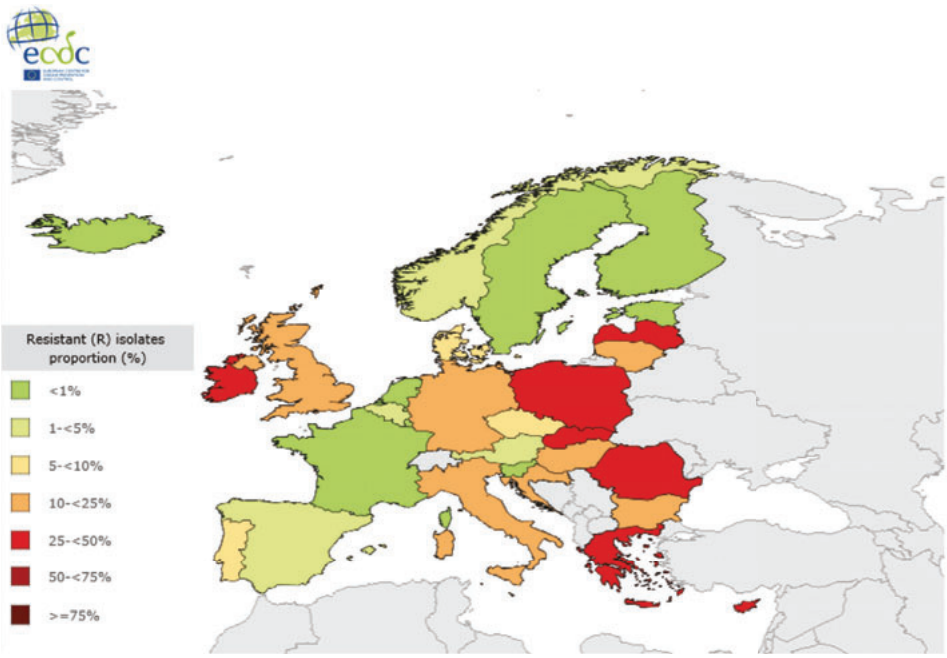
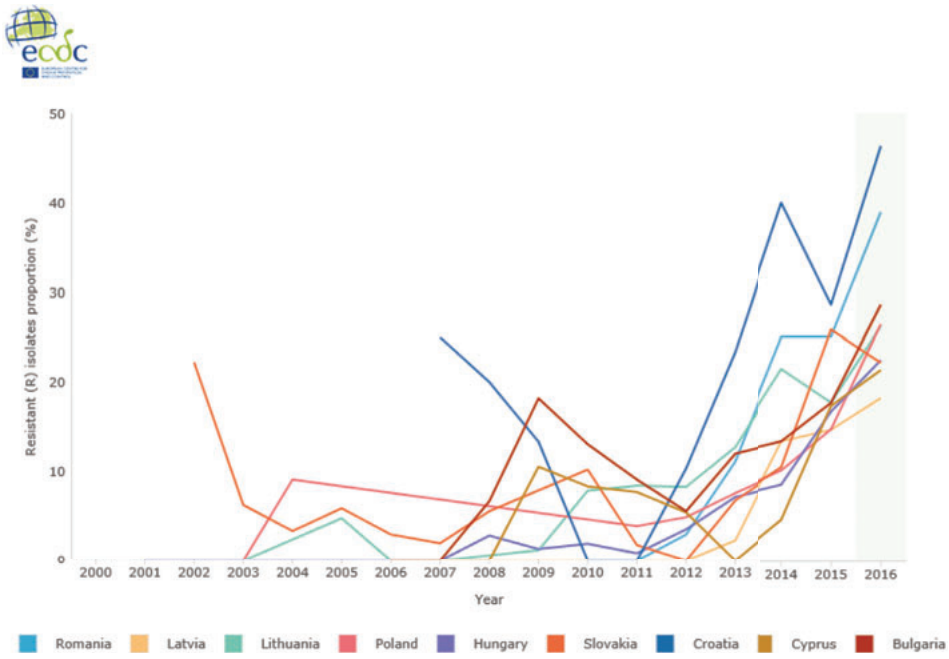


Figure 4: Data from the ECDC Surveillance Atlas- Antimicrobial resistance. Showing vancomycin resistance proportion rates in *Enterococcus faecium* in Europe for 2016. Dataset provided by ECDC based on data provided by WHO and Ministries of Health from the affected countries.



Australia reports a similar increasing trend in VRE prevalence as in many countries in Europe. The AESOP reports show a steadily increase in VREfm from 36.5% in 2010, to 46.1% in 2014 [28, 70-72]. The majority of isolates were grouped into CC-17, where ST203 has an predominant place across most regions of Australia since 2010. Other reported predominant sequence types are ST17, ST555 and the rapidly increasing ST796, largely replacing ST203 [73]. Especially VanB-type VRE dominated the epidemiology of VRE in Australia, but in recent years VanA-type VRE emerged. Whereas *vanA* VREfm was rarely detected in 2010, in 2014 18.5% of the VREfm bacteremia isolates harbored the *vanA* gene [28] . Interestingly, the recent emergence of *vanA* VREfm was associated with several STs and *vanA*-containing plasmids. This suggests multiple introductions of the *vanA* operon into the circulating *E. faecium* clones. It has been suggested that this could be due to sources in the community, or through introduction by health-care associated travel from oversea [74, 75].

Figure 5: Data from the ECDC Surveillance Atlas- Antimicrobial resistance. Showing the rapid increase in vancomycin resistance proportion rates in *Enterococcus faecium* for selected (Eastern) European countries: Romania. Latvia. Lithuania. Poland. Hungary. Slovakia. Croatia. Cyprus and Bulgaria. from 2002-2016. Dataset provided by ECDC based on data provided by WHO and Ministries of Health from the affected countries.



Worrying reports about the emergence of VREfm are also coming from countries in Asia, South-America, Africa, Russia and the Middle-East [76-81] underlining spread of successful HA- *E. faecium* lineages worldwide.

Altogether, nosocomial VRE lineages are arising in hospitals over all continents. The incorporation of MGEs such as *vanB*-carrying transposons into successful circulating HA-VSEfm lineages seems to be a significant factor in the emergence of *vanB* VREfm. This can occur via the exchange of large chromosomal fragments, including Tn*1549*, between *vanB* VREfm and VSEfm [64, 82]. Incidentally, de novo acquisition of *Tn1549* from anaerobic gut microbiota to VSEfm may occur [83]. If these events are subsequently followed by clonal expansion, this could lead to an increase in numbers of *vanB* VREfm [83] (Zhou et al. accepted). The success factors for the rapid dissemination of *E. faecium*, however, are probably not only the acquisition of antibiotic resistance and virulence genes, but may also include more specific adaptations to hospital conditions (discussed below).

THE EVOLUTION OF *ENTEROCOCCUS FAECIUM* SHAPED BY INFECTION CONTROL MEASURES AND DIAGNOSTICS IN MODERN HOSPITALS

E. faecium has many challenges to overcome to remain endemic in hospital environments. The spread of highly resistant microorganisms (HRMOs) in hospitals in general is limited by hand hygiene precautions and disinfection of patient rooms and medical equipment. In addition, the spread can be stopped by contact isolation of patients and targeted antibiotic treatment once HRMOs are detected. HRMOs that are not detected may spread in the hospital without being noticed and thereby have an advantage over detectable phenotypes. Diagnostic strategies may therefore have a selective role in the emergence of hospital lineages. In fact, the ability to evade diagnostics may be considered as a success factor in the emergence of VREfm lineages [84].

Diagnostic evasion mechanism

Several evasion mechanisms in the detection of VRE, VanA-type as well as VanB-type, have been reported in literature. These phenotypes of VRE, that evade detection by standard recommended methods for detection of glycopeptide resistance in *E. faecium* such as MIC determination, disk diffusion and the breakpoint agar method [85], are involved in uncontrolled outbreaks of VRE.

Detection of *vanB* VRE can be challenging since vancomycin MIC values can range from ≤0.5mg/L to ≥32mg/L in routine automatic susceptibility testing (AST) systems like Vitek2 (bioMérieux) and Phoenix [84]. Especially those strains that are tested vancomycin-susceptible according to the EUCAST susceptibility breakpoint of ≤4mg/L [86] are at risk to create an uncontrolled spread in healthcare settings. Percentages of these *vanB*-positive low-level vancomycin resistant VRE strains can range from 24.5%-55% in hospital outbreak settings [84, 87]. Moreover, the sensitivity of VRE screening declines as the fecal VRE density decreases and if media are assessed at 24 hours instead of 48 hours [88]. Therefore, it has been advised to screen multiple rectal swabs (up to four or five rectal swabs) to detect > 90-95% of the carriers [89, 90]. At last, direct detection of *vanB* carriage by molecular detection can be compromised by many false positive results due to *vanB* genes in non-enterococcal anaerobic bacteria present in the gut [91-95]. For this, in a PCR-based VRE screening, the use of enriched inoculated broth containing anti-anaerobic antibiotics, combined with adjusted cut-off cycle threshold (Ct)-values might be a useful and rapid tool in the detection of *vanB* VRE carriage [96].

Pitfalls in detecting *vanA* VRE can be due to an altered phenotype of *vanA* VRE. The expression of teicoplanin resistance can be heterogeneous conferring into a VanB-phenotype [97]. The presence of *vanS* (sensor) and *vanR* (regulator) genes in the *vanA* cassette are essential for the expression of glycopeptide resistance. Some isolates can test vancomycin and teicoplanin susceptible because of major nucleotide deletions or even absence of *vanS* and *vanR* genes in the *vanA* transposon [98, 99] or due to insertion of *IS* elements in the coding regions of the *vanA* transposon [100]. These *vanA*-positive enterococci, phenotypically susceptible to vancomycin are also termed as vancomycin-variable enterococci (VVE) [101]. These VVE are in stealth mode and are at risk to spread unnoticeably. In case of major deletions or complete absence of *vanS/R* genes and thus non-functional, strains will probably not revert under vancomycin therapy. However, in case of small deletions in the *vanR/S* region or if *vanA* VRE is silenced by *IS* elements, the strains can revert into vancomycin resistant strains upon vancomycin therapy [100, 102] which can lead to treatment failure.

In addition, VRE may evade detection by molecular diagnostics because multiple distinct gene clusters may confer resistance to vancomycin. Nowadays, nine different *van* genes in enterococci have been described (*vanA, B, C, D, E, G, L, M,* and *N*) [103-106]. Since VRE outbreaks are mainly due to *vanA* and/or *vanB* VREfm [41, 107], PCR-based methods most often only target *vanA* and *vanB*, but not the other types of *van* genes. VRE harboring mobile genetic islands with *vanD* are sporadically found in patients, but thus far no dissemination of these islands has been detected [108]**.** However, its prevalence may be underreported since the *vanD* gene is not detected by routine molecular diagnostics.

Infection control measures

Next to diagnostic evasion, survival in the environment by high tenacity and resistance to disinfection procedures are important adaptive traits of VRE hospital lineages. Enterococci are highly-tenacious microorganisms by nature. Compared to their ancestors, enterococci acquired traits that have led to an increased tolerance to desiccation and starvation, which make them resistant to environmental stresses similar to those occurring in modern hospitals [1]. Indeed, VRE can even survive for many years in the hospital environment [109, 110]. Enterococci are therefore excellent indicators of hygiene: culturing of surface swabs makes environmental contamination visible [111]. As a consequence, transmission of enterococci not only occurs directly through contaminated hands of health care workers, patients, or visitors, but also indirectly through contaminated environmental contaminated surfaces [6].

Enterococci are often isolated from high-contact points such as bed rails, over-bed tables, blood-pressure cuffs, alarm buttons, toilet seats and door handles [112]. Contaminated surfaces represent hidden reservoirs, from which enterococci may re-emerge and colonize patients that are subsequently admitted to the contaminate room [109, 113]. In attempts to eradicate persistent reservoirs with VRE, intensified cleaning measures like targeted cleaning of environmental surfaces using high concentrations of sodium chloride or decontamination with hydrogen peroxide vapor (HPV) should be used [114, 115].

Enterococci can be tolerant to low concentrations of chemicals such as alcohol and chlorine [116]. Worryingly, especially successful emerging *E. faecium* clones seem to be able to develop alcohol tolerance over time. After the systematically introduction of alcohol-based hand rubs in Australian hospitals, the use of hand alcohols increased during 2001-2015. Interestingly, tested HA *E. faecium* strains from hospitals in Australia isolated between 1998 and 2015, showed a significant increase in isopropanolol tolerance towards recently circulating emerging strains [117]. Although the alcohol tolerance experiments were established with a concentration of 23%, lower than the 70% which is used in hand alcohols, these tolerant *E. faecium* isolates did survive better than less tolerant isolates after 70% isopropanolol surface disinfection. This again is an example of how *E. faecium* can easily adapt to environmental changes such as increased use of hand alcohols. Inter-individual varieties between healthcare workers in hand hygiene compliance could lead to a variety in VREfm reductions on hands. In case of limited reduction, there might be an unforeseen spread of VREfm.

In addition to high survival to desiccation and starvation, heat-resistance is an important characteristic of enterococci. In the early days of microbiology, the exceptional heat-resistance of enterococci had already been reported in studies investigating pasteurization of dairy products [118]. A study comparing heat resistance of VSE versus VRE showed that some vancomycin-resistant isolates even survived exposure to 80 degrees Celsius for several minutes [116]. This is of particular relevance for infection control practices. For instance, disinfection procedures of bedpans regularly include heating at 80 degrees for one minute.

Several infection prevention strategies have been advised by the CDC Hospital Infection Control Practices Advisory Committee (HICPAC) in controlling VRE. This includes prudent use of vancomycin, education programs for hospital staff, early detection and reporting of VRE by clinical microbiology laboratories and isolation precautions and implementation of infection-control measures to prevent transmission of VRE, including contact isolation for VRE-positive patients [119]. It is difficult to conclude which infection prevention measure has the highest impact. The implementation of hand hygiene and decreasing environmental contamination

by enforced cleaning measures seem to have a significant impact on reducing the spread of VRE [120, 121]. However, single infection prevention measures often fail to have a real effect on reducing VRE rates. A multifaceted program implementing several guidelines, such as advised by the HICPAC, are therefore often needed to observe a clear reduction in VRE rates [122, 123].

Antibiotic use, especially anti-anaerobic antibiotics such as metronidazol, vancomycin and cephalosporin are risk factors for VRE acquisition [34, 124-126]. Moreover, ceftriaxone usage has been associated with blood stream infections with VRE [127]. Thus, stringent use of antibiotics to reduce the selective pressure is important and has successfully been applied in controlling ongoing VRE outbreaks [128, 129]

As a patient with an infection caused by VRE could be the tip of an iceberg [130] active surveillance cultures to detect VRE-carriage in patients at high-risk units [89] or patients transferred from foreign countries with high VRE prevalence in another important infection prevention measure. As noted earlier, detection of VRE can be complicated. Moreover, several rectal samples, on average four to five, are needed to detect the majority of carriers (>90-95%) [89, 90].

Molecular typing of *Enterococcus faecium*

In VRE outbreak investigations, rapid and accurate typing is required to investigate the genetic relatedness between patients' isolates. This information is essential to demonstrate nosocomial transmission and whether it is needed to enhance infection prevention measures. Rapid typing followed by infection prevention measures can lead to rapid control of nosocomial spread [131]. In Table 1 we summarized common used VRE typing methods including important characteristics; reproducibility, ease of performance, data interpretation, ease of data exchange and costs. WGS is increasingly used in clinical microbiology and outbreak analysis [132], including VRE outbreaks [63, 133, 134] and provides the highest discriminatory power herein. In addition, WGS offers the possibilities to perform pan-genome analysis to even enhance the assessment of genetic relatedness [135]. Additionally, a wide range of information can be extracted from WGS data such as MLST, core-genome (cg) MLST, whole-genome (wg)MLST data, virulence factors, resistance genes, plasmids and other genetic markers. However, there are some challenges to overcome to make it more accessible in daily routine clinical microbiology and outbreak analysis. Most important are the standardization and validation of procedures [136] and the interpretation of data [137]. The ease of data interpretation depends on the type of analysis to perform and which tools are available [132, 138, 139]. For example, cgMLST data can easily be extracted from WGS data by several

Table 1: Common used VRE typing methods including important characteristics; reproducibility, ease of performance, data interpretation, ease of data exchange and costs.

Method	MLVA	MLST	PFGE	cgMLST	WGS	Transposon analysis
Principle	Fragment length of variable tandem repeat loci	Sequencing of seven housekeeping genes	DNA based macro restriction analysis	Genome-wide gene-by-gene approach of 1423 genes on allelic level	Whole genome analysis	Analysis of transposon content and integration
Reproducibility	High	High	Medium	Excellent	Excellent	Excellent
Ease of performance	Very easy	Easy	Laborious	Easy	Easy	Easy
Data interpretation	Easy-moderate	Easy	Difficult	Easy	Various	Moderate
Ease of data exchange	Easy	Easy	Difficult	Easy	Possible	Possible
Costs	Low	Medium	Medium	High, extracted from WGS	High	High, extracted from WGS
Discriminatory power	Low	Medium	High	Excellent	Excellent	Additional

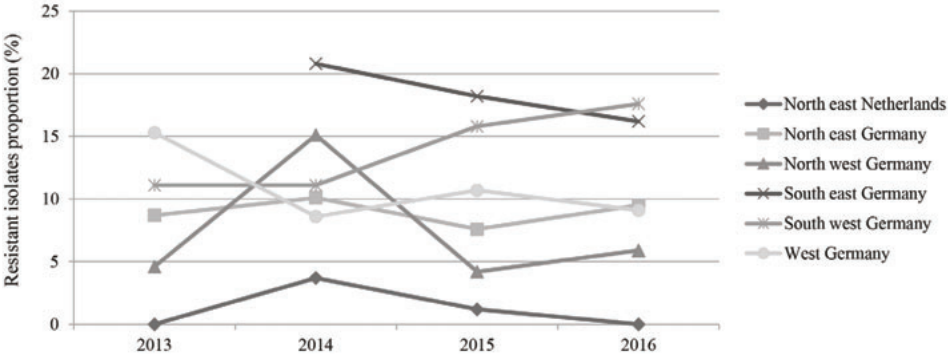
MLVA=Multiple Locus Variable Number of Tandem Repeat Analysis, MLST=Multi-locus Sequence Typing, PFGE=Pulsed-field gel electrophoresis, cgMLST=core-genome MLST, WGS=whole-genome sequencing.

in-house and commercially software packages. Compared to MLST, cgMLST has a higher discriminatory power in distinguishing genetically related and unrelated *E. faecium* isolates [140]. The advantage of cgMLST over SNP-based methods is that the data can be easily compared, stored and shared in web-based databases that can be interrogated (http://www.cgmlst.org/ncs/schema/991893/). Importantly, if VRE outbreaks are caused by the horizontal transfer MGEs encoding vancomycin-resistance, studying the molecular epidemiology of these MGEs by specifically analyzing variation of transposons encoding *vanA* or *vanB* gene clusters is essential and will enhance the resolution of used typing methods. The use of WGS to study the molecular epidemiology of VRE will also facilitate detailed analysis of variation in these vancomycin-resistance encoding transposons. This will provide the best insight in VRE outbreaks, elucidating the complex transmission routes [83] (Zhou et al. accepted).

FUTURE PERSPECTIVES:

In the upcoming years, it will be a challenge to withstand the spread of VREfm. A rapid and ongoing emergence of VREfm is observed in countries in Central and Eastern Europe since 2010. Large regional differences have been observed in this rise of VREfm infections, even within countries. This is underlined by the regional differences in VREfm proportions in German and Dutch regions (Figure 6). In 2016, the lowest proportion in Germany was reported in the region of North-West Germany (5.9%), which is in contrast with the proportion in the North-East (9.5%), South-East (16.2%), and South-West (17.6%) [141]. The proportion of VRE in the Dutch Northern-East region bordering with North-West Germany remained very low between 2013 and 2016 (Figure 6). Among these two regions, collaborative cross-border INTERREG-projects focusing on prevention of the spread of highly-resistant microorganisms are ongoing. Although there is no conclusive explanation for the variations in the German regions, surveillance and outbreak management strategies, antibiotic stewardship policies [142], and differences in patient traffic from high prevalence countries may be important factors. In some countries, VRE infection control policies only focus on patients with infections, while in others patients belonging to high-risk populations are also screened for VREfm-carriage as recommended by HICPAC [119].

Figure 6: Showing the proportion of vancomycin resistant isolates (%) in *Enterococcus faecium* for different regions in Germany (North-East. North-West. South-East. South-West and West) and North-East Netherlands. For South East Germany no data were available for 2013.



VRE infections are commonly preceded by VRE-carriage, as described in our review. Early detection of carriage may prevent the spread and reduce the number infections. In the Netherlands, for example, there have been many outbreaks with patients carrying VRE. These outbreaks were controlled in an early phase, and thereby the proportion of infections with VRE is still low in the Netherlands. Thus, if the goal of a hospital is to prevent VREfm infections, special attention is required to reduce the VREfm spread by screening for VREfm-carriage. Other important factors are the role of hospital environment contamination by VREfm and the challenges in detection and typing of VREfm. To this end, we summarize recommendations described in literature and/or by guidelines (Table 2). Many of the recommendations follow directly from the traits of *E. faecium* as we reviewed. So far, these recommendations have shown to be successful in the control of VREfm in the Netherlands. However, these measures are very expensive and require a lot of effort of medical (molecular) microbiologists and infection control specialists [129]. VRE diagnostics are difficult in particular, as described in this review. Innovations in the detection and typing of VREfm are required to overcome these difficulties. Development of better selective media, PCRs with higher specificity, or rapid point of care tests are needed to detect VRE more efficiently. A promising development is the use of clone-specific PCRs, which might be helpful to detect and control VREfm outbreaks caused by specific clones [143]. This method combines typing and detection in a rapid and cost-effective manner [144].

It is a point of debate whether these efforts are worthwhile to control the spread of VREfm. The attributable mortality of the currently successful VREfm lineages are mainly

due to inappropriate (empirical) antibiotics rather than additional virulence of vancomycin resistance [145-147]. However, treatment options are limited in VREfm, since *E. faecium* is intrinsically resistant to many antibiotic classes. Resistance to several last-line enterococcal drugs like linezolid, daptomycin, tigecycline, and quinopristin-dalfopristin have already emerged [148-151]. Therefore, further research and development of antimicrobial targets for the treatment of MDR *E. faecium* is needed [152]. Development of new antibiotics is very expensive, takes a lot of time, and there is a risk on rapid development of resistance to these new drugs as well. In the meantime, it is important to be prudent with the current antibiotics available, and optimize adherence to hygiene precautions to prevent the patient to patient spread of VRE resistant to these last-line antibiotics. For this purpose, it may be wise to reduce the spread of VREfm by surveillance on VREfm carriage in high risk populations. In many hospitals this might be difficult to realize. Capacity building programs and structural financial support for hospitals would be needed to implement efficient nosocomial screening on VREfm-carriage and subsequent infection control measures. Cross-border collaborations may prove useful in the implementation of such programs, and have previously shown to be successful in the decrease in MRSA prevalence in the Dutch-German Euregion [153].

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Table 2: Essential traits of *Enterococcus faecium* and their translation into implications and practical recommendations on the laboratory and infection control level.

Traits of *Enterococcus faecium*	Implications for infection control	Recommendations
High tenacity and intrinsic resistance environmental stress	– Prolonged survival in hospital environment. – High survival to desiccation and starvation. – Resistance to heat and disinfection procedures.	– Intensified cleaning procedures, including intensified cleaning procedures and prolonged disinfection procedures [110, 114, 116]. – Implementation of infection-control measures to prevent transmission of VRE, including isolation precautions for VRE-positive patients [119]. – Education programs for hospital staff, including hand hygiene to prevent further transmission [119]. – Environmental cultures in (uncontrolled) VRE outbreaks and surveillance cultures after disinfections.
Intrinsic resistance antibiotics	– Outgrowth under antibiotic pressure. – Prone to become pan-resistant.	– Antibiotic stewardship, especially prudent use of vancomycin (reduce emergence of VRE) [119] and metronidazole (reduce outgrowth of VRE) [32, 37]. – Surveillance and controlling of VRE-carriage in hospitals [119].
Genome plasticity	– Continuously adaptation and refinement in response to environmental changes. – Development of resistance to newer antibiotics and disinfectants in the future.	– Continuous awareness and surveillance to detect resistance to newer antibiotics and disinfectants. – Further research and development of antimicrobial targets for the treatment of MDR *E. faecium* is needed [152].

Traits of *Enterococcus faecium*	Implications for infection control	Recommendations
Diagnostic evasion	– Phenotypes of evolutionary successful HA VRE lineages that evade detection by standard recommended methods for detection of glycopeptide resistance in *E. faecium* – Difficulties in detecting VRE-carriage due to low fecal densities	– Active surveillance cultures to detect VRE-carriage in patients at high-risk units or patients transferred from foreign countries with high VRE prevalence [119]. – Multiple rectal samples (four to five), are needed to detect the majority of carriers (>90-95%) [89, 90]. – Get knowledge of the local epidemiology of VRE and vancomycin MICs in own hospital. – Early and accurate detection and reporting of VRE by clinical microbiology laboratories [119]. – For rapid screening of VRE carriage, a combination of selective enrichment broths and molecular detection increases the sensitivity [96]. – Use of selective (chromogenic) agar [154]. – Vancomycin disk diffusion according to EUCAST [155]. – Genotypic testing of invasive vancomycin-susceptible enterococci by PCR [84].
Common origin of hospital lineages in early 20th century (CC-17)	– Typing difficulties during VRE outbreaks.	– Rapid and accurate typing is needed to take adequate infection prevention measures. – Preferably a highly discriminatory typing method like cgMLST or WGS, ideally combined with transposon analysis

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