Enterococcus faecium: from evolutionary insights to practical interventions

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The research was partly supported by:

- the INTERREG V A funded project EurHealth-1Health (202085), which is part of a Dutch-German cross-border network supported by the European Union, the Dutch Ministry of Health, Welfare and Sport (VWS), the Ministry of Economy, Innovation, Digitalisation and Energy of the German Federal State of North Rhine-Westphalia and the German Federal State of Lower Saxony
- the INTERREG IV A funded project EurSafety Health-net (III-1-02=73) part of a Dutch-German cross-border network supported by the European Commission, the German Federal States of Nordrhein-Westfalen and Niedersachsen, and the Dutch provinces of Overijssel, Gelderland, and Limburg.

The printing of this thesis was financially supported by the Division "Microbial Typing" of the KNVM, the Graduate School of Medical Sciences and the INTERREG IV/V A projects EurHealth-1Health and EurSafety Health-net. Their support is highly appreciated.

ISBN: 978-94-034-1130-9 Printed version

ISBN: 978-94-034-1129-3 E-book

Cover design: Xuewei Zhou

Layout and design: Jules Verkade, persoonlijkproefschrift.nl

Printing: Ridderprint BV | www.ridderprint.nl

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Enterococcus faecium: from evolutionary insights to practical interventions

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. E. Sterken
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 19 december 2018 om 14.30 uur

door

Xue Wei Zhou

geboren op 6 maart 1987
te Leeuwarden

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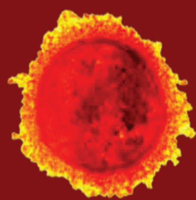
TABLE OF CONTENTS

Chapter 1	Introduction	7
Chapter 2	*Enterococcus faecium*: from fundamental insights to practical recommendations for infection control and microbiological diagnostics *Submitted*	15
Chapter 3	Epidemiology of Extended Spectrum β-lactamase-producing *E. coli* and vancomycin-resistant enterococci in the Northern Dutch-German cross- border region *Frontiers in Microbiology-Evolutionary and Genomic Microbiology 2017 Oct 5;8:1914*	49
Chapter 4	Algorithm for pre-emptive glycopeptide treatment in patients with haematologic malignancies and an *Enterococcus faecium* bloodstream infection *Antimicrobial Resistance and Infection Control 2013 Sep 11;2(1):24*	79
Chapter 5	Evaluation of the Xpert *vanA/vanB* assay using enriched inoculated broths for the direct detection of *vanB* VRE *Journal of Clinical Microbiology 2014 Dec;52(12):4293-7*	103
Chapter 6	Diagnostic evasion of highly-resistant microorganisms: a critical factor in nosocomial outbreaks *Frontiers in Microbiology-Antimicrobials, Resistance and Chemotherapy 2017 Nov 3;8:2128*	115
Chapter 7	Elucidating vancomycin-resistant *Enterococcus faecium* outbreaks: the role of clonal spread and movement of mobile genetic elements. *Accepted for publication in Journal of Antimicrobial Chemotherapy*	129
Chapter 8	Summary, conclusion & discussion and future perspectives	151
Chapter 9	Nederlandse samenvatting Dankwoord Biografie/Biography	165



Introduction

1

INTRODUCTION

Enterococci are facultative anaerobic gram-positive cocci which can be found as commensals in the gastrointestinal tract of humans, other mammals, birds, insects and reptiles [1]. The genus *Enterococcus* has originated around 425-500 million years ago. Around this time of animal terrestrialization, enterococci emerged from their ancestor *Vagococcus*. Vagococci diverged from *Carnobacteriaceae*, which resided in marine environments [2, 3]. Vagococci were thereby adapted to salty habitats. These environmental conditions predisposed this genus to colonize the gastro-intestinal tracts of animals, in which the bacteria are exposed to bile salts. Vagococci were already able to colonize ecologies with high levels of bile, a characteristic feature in enterococci [4]. As a consequence of the migration of animals from water to land, the environmental conditions for enterococci changed. When the bacteria were outside the host in the environment on land, they were exposed to dry conditions and starvation, in contrast to the humid coastal conditions of the previous habitat. These conditions selected for species with highest tenacity. Compared to their ancestor, enterococci are significantly enriched in the cell wall modification and de novo purine biosynthesis, forming cell wall components that increases its integrity [5, 6]. These functions are related to environmental stress responses. The thickened cell wall protects the enterococci against desiccation and starvation. The thick and impermeable cell wall also resulted in non-permeability for many antibiotic classes. Thereby, enterococci are intrinsically resistant to cephalosporins, low-level aminoglycosides and clindamycin [1]. In addition to their intrinsic antibiotic resistances, they can easily acquire antibiotic resistance genes [7] of which vancomycin resistance is clinically most relevant.

Subsequently, the evolution of the animal hosts had a great influence on the evolution of enterococci. Utilization of carbohydrates provided by the host has been, and still is a major driver in enterococcal speciation. Large gains of genes for carbohydrate metabolic pathways are seen in the emergence and proliferation of enterococci which parallels the radiation of hosts [4]. The availability of uric acid in the hosts' gut, and the ability of enterococci to metabolize this carbon source, is of particular interest. Biofilm formation can be triggered by the metabolites formed in uric acid degradation [8]. This biofilm formation is suggested to increase the virulence of enterococci in uricotelic hosts [4].

Enterococci are generally considered as non- or low-pathogenic micro-organisms and mainly being clinically relevant in case of hospital associated (HA) infections. Around the 1970s and 1980s, enterococci emerged as a leading cause of HA infections mainly due to *E. faecalis* and *E. faecium*. Especially *E. faecium* seemed to rapidly emerge as a nosocomial

pathogen worldwide. Indeed, the worldwide emergence of vancomycin resistant enterococci (VRE) is largely caused by the rise of vancomycin resistant *E. faecium* (VREfm) [9, 10]. The successful *E. faecium* and VREfm lineages that are circulating in hospitals are characterized by ampicillin resistance, pathogenicity islands and are associated with hospital outbreaks [11]. Studies have shown that these HA *E. faecium* isolates acquired a number of traits making them successful in the hospital environment. These strains contain more antibiotic resistance and virulence genes enhancing biofilm formation and colonisation [12].

Within a short period of time, *E. faecium* has rapidly evolved as a successful nosocomial pathogen. By ease they have withstand and adapted to environmental changes in life, such as human urbanization, antibiotic pressure and the modern hospital environment. Further insight in the successful evolution of *E. faecium* is reviewed in Chapter 2 of this thesis.

Scope and outline of this thesis

The first chapters of this thesis aim to gain insight in the evolution and epidemiology of *E. faecium* (Chapters 2, 3 and 6). From these insights, this thesis proceeds to innovations that have value for patient care. The rapid emergence of hospital lineages imposes challenges for controlling, detecting and typing of VRE. To overcome these challenges, antibiotic stewardship strategies and diagnostic innovations using molecular techniques are required. This thesis describes such innovations, including model-based antibiotic prescription guidance, tailor made diagnostic tools for (vancomycin resistant) *E. faecium*, targeted VREfm infection prevention measures and highly discriminating typing methods in VREfm outbreak investigations (Chapters 2 and 4-7).

Chapter 2 provides an overview of the background and historical evolution of *E. faecium*. We aimed to describe which successful traits and conditions have had a high impact on *E. faecium*, becoming a successful nosocomial pathogen. The increase of *E. faecium* infections in hospitals worldwide as well as the subsequent emergence and epidemiological background of vancomycin resistant *E. faecium* (VREfm) will be reviewed. Additionally, the role of current modern laboratory diagnostics and infection prevention measures in the emergence of VREfm will be discussed. Finally, 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 guidances.

The prevalence and molecular epidemiology of extended-spectrum β-lactamase-producing (ESBL)/plasmid AmpC (pAmpC) bacteria and HA *E. faecium* (including VRE) in the Northern Dutch-German cross-border region is described in **Chapter 3**. For this

purpose, a point-prevalence study was performed in hospitalized patients in the Northern Netherlands and North-West Germany. Also, healthy individuals from the Dutch community were screened. A genome-wide gene-by-gene typing approach was applied to study the molecular epidemiology of ESBL-*Escherichia coli* and VRE.

In **Chapter 4** of this thesis we aimed to identify certain risk factors for the development of an *E. faecium* bloodstream infection in patients with haematologic malignancies. Haematology patients have a high risk of an *E. faecium* bloodstream infection, but empirical therapy usually does not cover this bacterium. Antibiotic treatment of *E. faecium* includes glycopeptides such as vancomycin. However, prudent use of vancomycin is needed for the control of VRE. Therefore, we aimed to design a prediction model based on identified risk factors for *E. faecium* infections to corroborate the clinical decision to start glycopeptides pre-emptively in haematology patients.

Chapter 5 describes the evaluation of a PCR-based diagnostic method, the Xpert *vanA/vanB* assay, for the detection of *vanB* VRE carriage. This assay runs on a Cepheid GeneXpert system which is, after adding the clinical sample to a cartridge, fully automated combining DNA extraction, real-time PCR amplification and detection. Direct detection of *vanB* VRE on faecal samples is complicated due to the presence of non-enterococcal *vanB* genes from anaerobic gut bacteria. This could lead to many false-positive results. The assay was used on enriched broth, containing antibiotics selective for enterococci but suppressing anaerobes. Additionally, an adjusted cycle threshold (C_t) cut-off value was determined to optimize the accurate and rapid detection of *vanB* VRE.

In **Chapter 6** the diagnostic evasion of highly-resistant microorganism (HRMOs) as a critical factor in outbreaks is described. Various examples of resistance mechanisms in carbapenemase-producing Enterobacteriaceae (CPE), VRE, methicillin resistant *Staphylococcus aureus* (MRSA) and ESBL are given that result in evasion of detection by routine diagnostic approaches. For each HRMO, mechanisms and examples of national and international outbreaks are described. Next, we aimed to provide practical laboratory detection advices to overcome the diagnostic evasion for these HRMOs.

Chapter 7 shows the application of whole genome sequencing (WGS) in VREfm outbreak diagnostics. The dissemination of VREfm is due to both clonal spread and spread of mobile genetic elements (MGEs) such as transposons. We analysed VREfm outbreaks that occurred in the University Medical Center Groningen (UMCG) in 2014. For this purpose, all epidemiological data of patients carrying these VREfm, including patients' transfer data, were gathered. Representative isolates with WGS data available were typed by core-genome

multi-locus sequence typing (cgMLST). Additionally, *vanB*-carrying transposons of all sequenced isolates were characterised. By combining cgMLST, transposon characterization and epidemiological data, we aimed to elucidate the pathways of transmission of VREfm outbreaks.

Finally, a summary of the results of this thesis is given in **Chapter 8**. This chapter also gives the overall conclusion and discussion, pointing towards some future perspectives.

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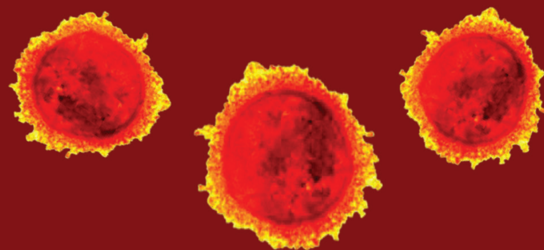

Enterococcus faecium: from fundamental insights to practical recommendations for infection control and microbiological diagnostics

2

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Keywords: *Enterococcus faecium*, VRE, evolution, diagnostics, infection control

Short title: *Enterococcus faecium* insights and microbiological recommendations

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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

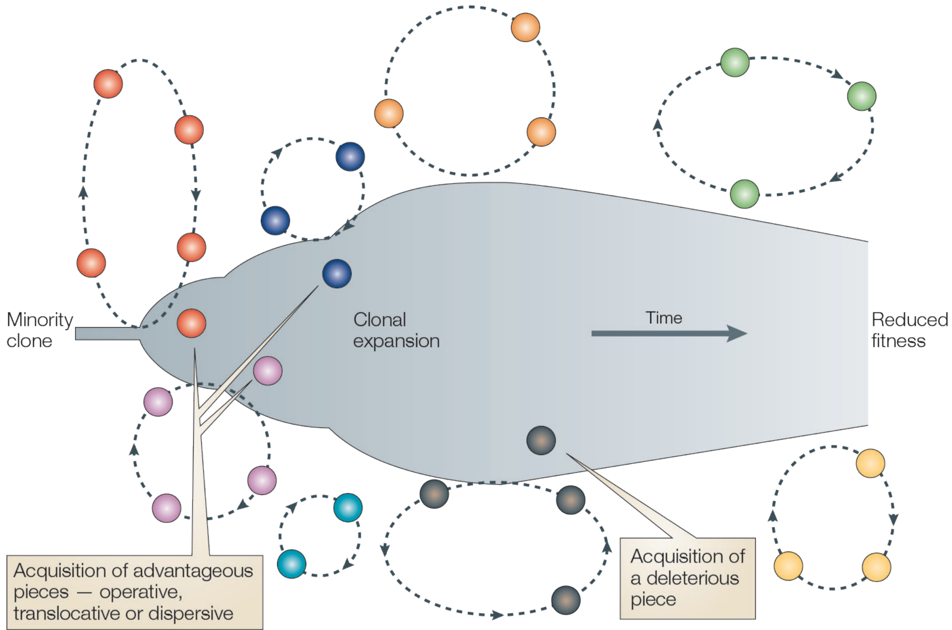
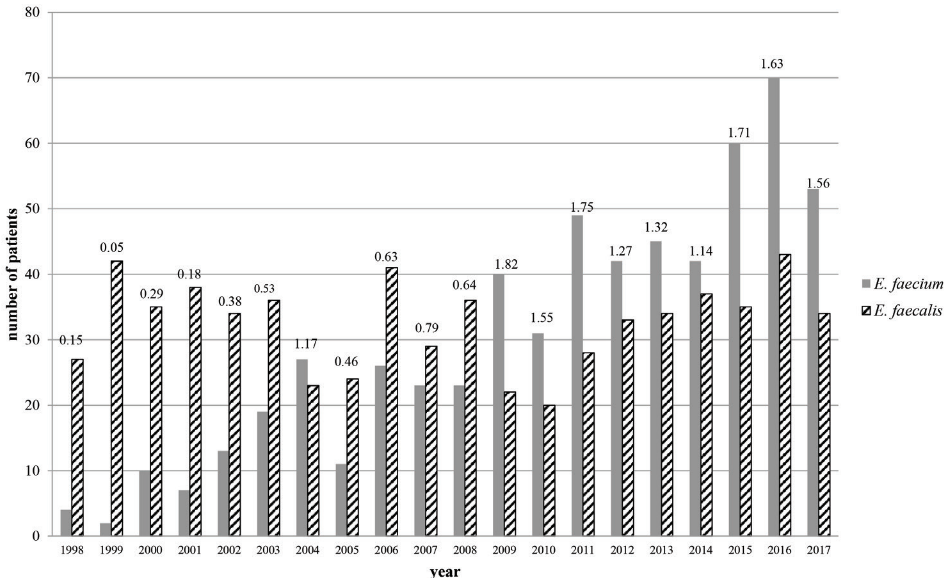


Figure 2: Number of patients with blood cultures with *E. faecium* and *E. faecalis* in individual patients and the *E. faecalis/E. faecium* ratio during 1998-2017 in the University Medical Center Groningen. The *E. faecium* to *E. faecalis* ratio changed approximately from 0.1 in 1998 to 1.6 in 2017.



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.

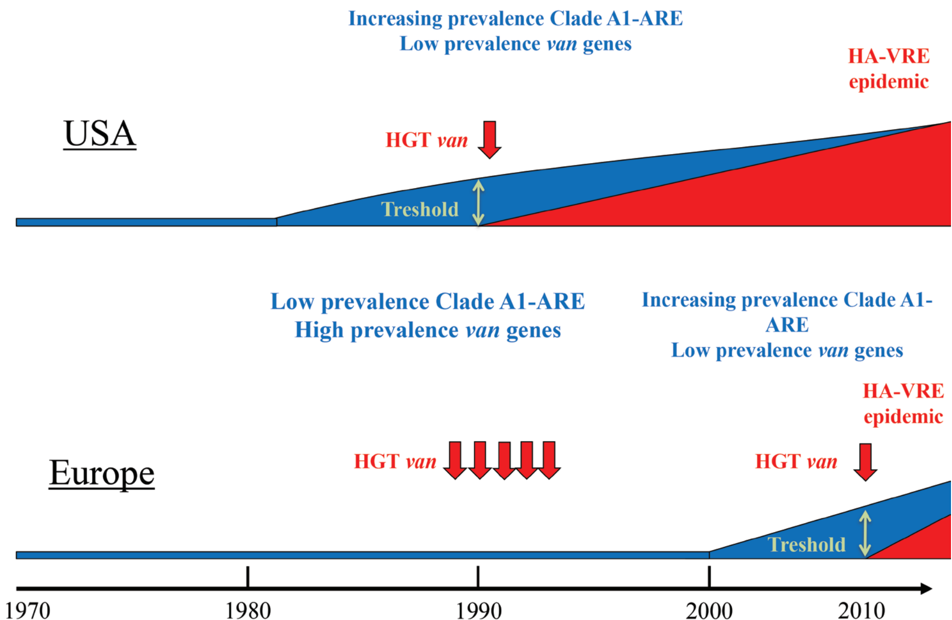
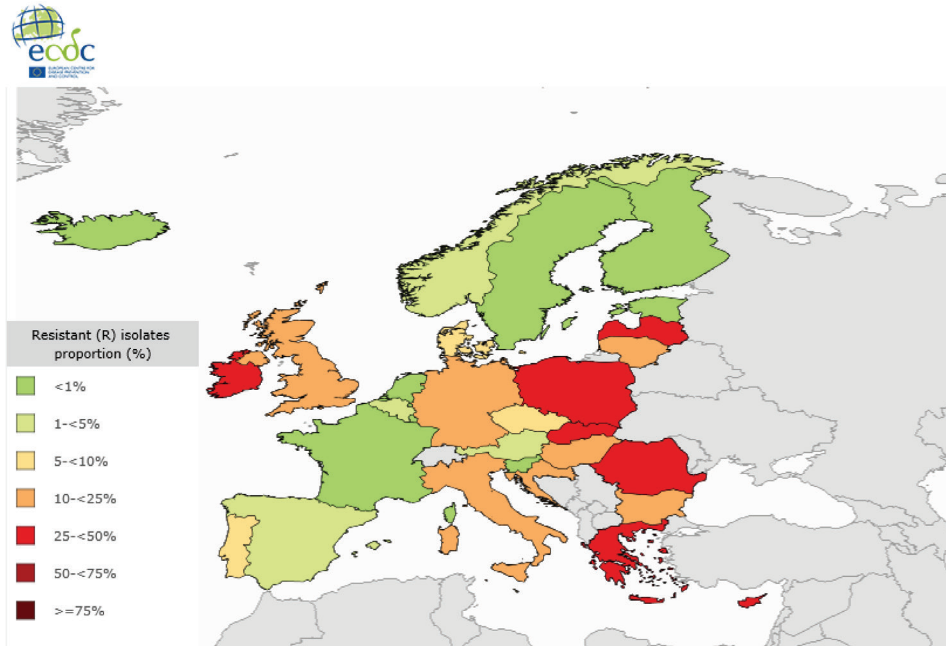
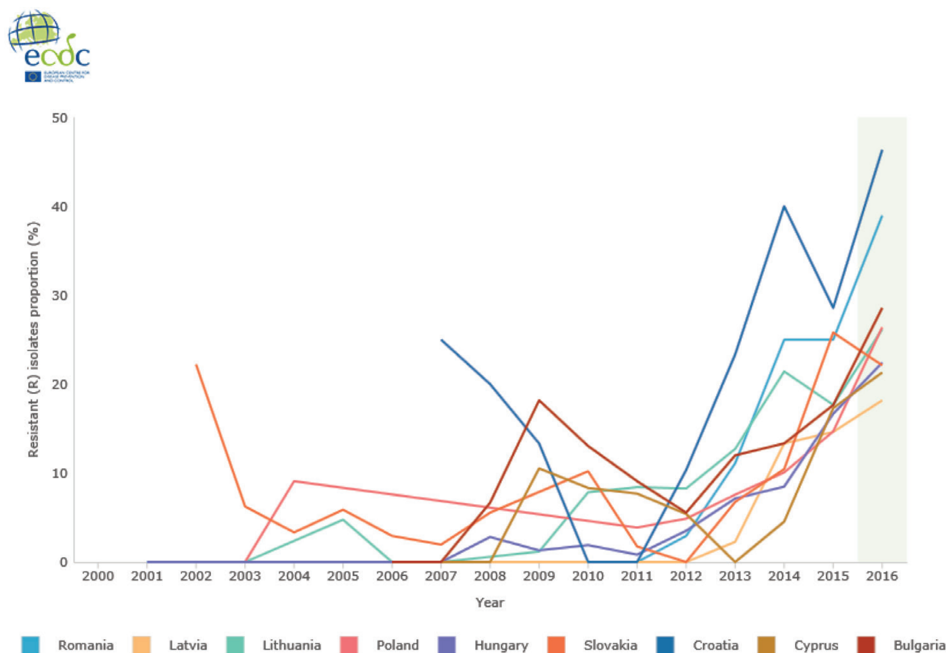


Figure 4: Data from the ECDC Surveillance Atlas- Antimicrobial resistance. Showing vancomycin resistance proportion rates in *Enterococcus faecium* in Europe for 2016. Dataset provided by ECDC based on data provided by WHO and Ministries of Health from the affected countries.



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

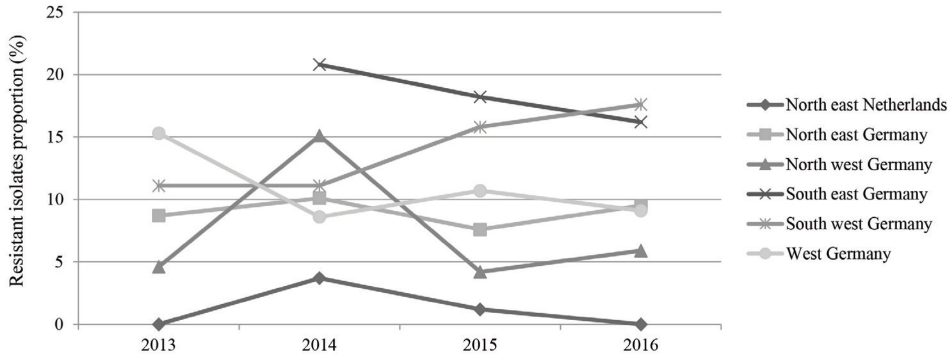
MLVA=Multiple Locus Variable Number of Tandem Repeat Analysis, MLST=Multi-locus Sequence Typing, PFGE=Pulsed-field gel electrophoresis, cgMLST=core-genome MLST, WGS=whole-genome sequencing.

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].

Acknowledgements

John Rossen consults for IDbyDNA. All other authors declare no conflicts of interest. IDbyDNA did not have any influence on interpretation of reviewed data and conclusions drawn, nor on drafting of the manuscript and no support was obtained from them.

This study was partly supported by the Interreg Va-funded project EurHealth-1Health (InterregVa/202085), part of a Dutch-German cross-border network supported by the European Union, the German Federal States of Nordrhein-Westfalen and Niedersachsen and the Dutch Ministry of Health, Wellbeing and Sport (VWS).

We would like to thank Mariëtte Lokate and Matthijs Berends for providing the data of the proportion of vancomycin resistant isolates (%) in *Enterococcus faecium* in the North-East Netherlands. We thank Jan Arends for providing the data of the positive blood cultures with *E. faecalis* and *E. faecium*.

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

2

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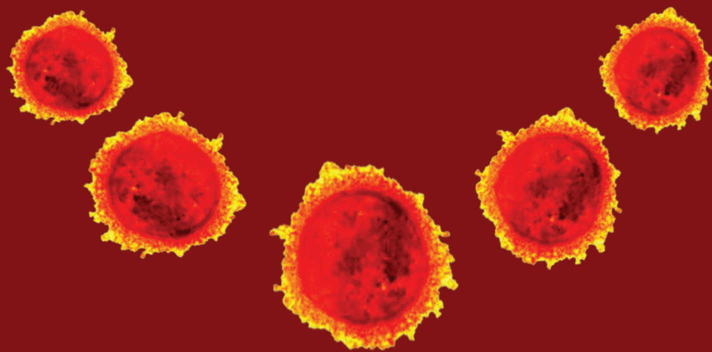

Epidemiology of Extended Spectrum β-lactamase-producing *E. coli* and vancomycin-resistant enterococci in the Northern Dutch-German cross- border region.

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Key words: WGS, cgMLST, VRE, ESBL, hospital, community, prevalence, cross-border research

Running title: Epidemiology of ESBL and VRE in hospitals and the community

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ABSTRACT

Objectives; To reveal the prevalence and epidemiology of Extended spectrum β-lactamase (ESBL)- and/or plasmid AmpC (pAmpC)- and carbapenemase (CP) producing *Enterobacteriaceae* and vancomycin resistant enterococci (VRE) across the Northern Dutch-German border region.

Methods; A point-prevalence study on ESBL/pAmpC,/CP producing *Enterobacteriaceae* and VRE was carried out in hospitalized patients in the Northern Netherlands (n=445, 2012-2013) and Germany (n=242, 2012). Healthy individuals from the Dutch community (n=400, 2010-2012) were also screened. In addition, a genome-wide gene-by-gene approach was applied to study the epidemiology of ESBL-*E. coli* and VRE.

Results; A total of 34 isolates from 27 patients (6.1%) admitted to Dutch hospitals were ESBL/pAmpC positive and 29 ESBL-*E. coli*, three pAmpC-*E. coli,* one ESBL-*E. cloacae* and one pAmpC-*P. mirabilis* were found. In the German hospital, 18 isolates (16 *E. coli* and 2 *K. pneumoniae*) from 17 patients (7.7%) were ESBL positive. In isolates from the hospitalized patients CTX-M-15 was the most frequently detected ESBL-gene. In the Dutch community, 11 individuals (2.75%) were ESBL/pAmpC positive: 10 ESBL *E. coli*, (CTX-M-1 being the most prevalent gene) and one pAmpC *E. coli*. Six Dutch (1.3%) and four German (3.9%) hospitalized patients were colonized with VRE. Genetic relatedness by core genome multi-locus sequence typing (cgMLST) was found between two ESBL- *E. coli* isolates from Dutch and German cross-border hospitals and between VRE isolates from different hospitals within the same region.

Conclusions; The prevalence of ESBL/pAmpC-*Enterobacteriaceae* was similar in hospitalized patients across the Dutch-German border region, whereas VRE prevalence was slightly higher on the German side. The overall prevalence of the studied pathogens was lower in the community than in hospitals in the Northern Netherlands. Cross-border transmission of ESBL-*E. coli* and VRE seems unlikely based on cgMLST analysis, though continuous monitoring is necessary to keep the epidemiology of resistant pathogens updated thereby helping to control their spread.

INTRODUCTION

International travel and patient care are risk factors for dissemination of bacteria including multidrug-resistant microorganisms (MDRO), such as Extended spectrum β-lactamase (ESBL) and carbapenemase (CP)-producing *Enterobacteriaceae* [1, 2], and vancomycin resistant enterococci (VRE). The prevalence of the latter has increased in the last years due to successful polyclonal subpopulations of hospital associated (HA) *E. faecium* (previously designated clonal complex CC17) and which are also associated with amoxicillin resistance (ARE) [3]. These populations are distinct from *E. faecium* isolates in the community and isolates from non-human sources [4, 5].

The Netherlands and Germany as bordering countries with possible transfer of patients between them, created a cooperative network to prevent the spread of MDRO and to harmonize guidelines in healthcare settings [1, 6]. Surveillance studies to monitor the prevalence, resistance patterns and molecular background of MDRO in hospitals and the community are essential to get insights into their epidemiology to implement infection prevention measures. Bacterial whole-genome sequencing (WGS) has been demonstrated to be very useful for epidemiological surveillance and detection of antimicrobial resistance [7]. The gene-by gene approach uses a defined set of genes to extract an allele-based profile which makes it scalable and portable between laboratories [8, 9]. A core genome multilocus sequence typing (cgMLST) scheme has been developed for *E. faecium* to distinguish between epidemiologically related and unrelated isolates [10]. Although there is no cgMLST scheme nor threshold publically approved yet for *E. coli*, there are several tools available that allow to define an *ad hoc* cgMLST.

The aim of this study was to perform a point-prevalence study on ESBL/plasmid mediated AmpC β-lactamase (pAmpC)/CP- *Enterobacteriaceae* and HA *E. faecium* (VRE and ARE) in hospitals in the Northern Dutch-German border region and to determine the predominant resistance genes. In addition, stool community samples from the Northern Netherlands were screened for the same resistant pathogens. A cgMLST was used to study hospital and cross border dissemination of ESBL-*E. coli* and VRE.

MATERIALS AND METHODS

Study design

A prospective point prevalence study was conducted in four of the largest hospitals (in total 3550 beds) in the Northern Netherlands between November 2012 and February 2013, covering a total population of approximately 2.85 million people. The Hospital Ethical Committee of the University Medical Center Groningen (UMCG) was informed and patients were approached to voluntarily participate in the study. Patients included in this study provided their written informed consent and a questionnaire concerning epidemiological and clinical data. The following high-risk wards for antibiotic resistant microorganisms were selected: intensive care units (ICU), vascular surgery, internal medicine haematology/oncology and dialysis wards (both for in- and outpatients). Gynaecology and neurology (low-risk wards) were also included for comparison. From the largest German university hospital in the same (border) region, patients from four ICUs, a surgical ward and a haematology/oncology ward were screened during October and November 2012 and included in the study. After consent agreement, all admitted patients from the studied wards were screened until completing a minimum of 100 samples per hospital.

The study in healthy people living in the the Northern Netherlands was conducted retrospectively, using control patients included in a previous case-control study on microorganisms causing gastroenteritis. Control subjects were patients attending their general practitioner for a variety of medical questions, but no gastrointestinal problems, in the period between August 2010 and December 2012 [11]. No prevalence study was performed in the community in Germany.

Sample collection

A total of 445 rectal swabs (Copan ESwab™) were taken from hospitalized patients (median age = 66 years, range 18-99 years) in the Northern Netherlands, 51.7% (n=230) from men and 48.3% (n=215) from women. A total of 328 (73.7%) patients were screened at high risk wards and 117 (26.3%) patients were screened at low risk wards (Table 1). In the German university hospital 242 patients (median age = 64 years, range 0-94 years) were included, 64.5% (n=156) men and 35.5% (n=86) women. Of these 242 patients, 140 were screened only for ESBL, 22 only for VRE and 80 for both. From the Dutch community study, 400 frozen faeces samples were included; 41% (n=164) from men, and 59% (n=236) from women, 12% of the samples were from children. The median age of the healthy individuals was 47.5 years (range 0-84 years).

Table 1: Distribution of ESBL/pAmpC producing *Enterobacteriaceae*, and amoxicillin and vancomycin resistant *E. faecium* among the different wards in Dutch hospitals.

Ward	ESBL/pAmpC producing *Enterobacteriaceae*	Amoxicillin resistant *E. faecium*	Vancomycin resistant *E. faecium*
High risk (n=328)	19 (5.8 %)	99 (30.2%)	6 (1.8%)
– Intensive care unit (n=102)	6 (5.9%)	31 (30.4%)	1 (1%)
– Vascular surgery (n=54)	6 (11.1%)	15 (27.8%)	1(1%)
– Internal medicine hematology/oncology (n=81)	1 (1.2%)	36 (44.4%)	2 (2.5%)
– Dialysis (n=91)	6 (6.6%)	17 (18.7%)	2 (2.2%)
Low risk (n=117)	8 (6.8%)	6 (5.1%)	0 (0%)
– Gynaecology (n=55)	3 (5.5%)	1 (1.8%)	0 (0%)
– Neurology (n=62)	5 (8.1%)	5 (8.1%)	0 (0%)
Total (n= 445)	27 (6.1%)	105 (23.6%)	6 (1.3%)

MICROBIOLOGICAL DETECTION, IDENTIFICATION AND SUSCEPTIBILITY TESTING

Dutch hospitals and retrospective Dutch community study

Rectal swabs (Dutch hospitalized patients) and approximately 50µg of faeces per sample (Dutch community patients) were enriched in selective broths: VRA broth containing BHI (brain heart infusion) with 20 mg/L amphoterin-B, 20 mg/L aztreonam, 20 mg/L colistin and 16mg/L amoxicillin and TSB-VC broth containing tryptic soy broth with 8 mg/L vancomycin and 0.25 mg/L cefotaxim. Both broths were incubated for 24h at 35 °C +/-1°C. Subsequently, 10µL of VRA broth was subcultured on VRE Brilliance agar (Oxoid®) and BMEG-2 agar (blood agar containing 64 mg/L meropenem, 2 mg/L gentamicin, 10 mg/L oxacillin and 20 mg/L amphotericin-B) for identification of VRE and all ARE, respectively. Ten µL of TSB-VC broth was subcultured onto ME/CF/CX comparted plates, containing iso-sensitest agar with 1 mg/L meropenem, 1 mg/L ceftazidim, or 1 mg/L cefotaxim respectively, plus 20 mg/L vancomycin and 20 mg/L amphotericin-B (Mediaproducts, Groningen), for selection of ESBL/pAmpC/CP- producing bacteria. Plates were incubated for 24h at 35°C +/-1°C, except for VRE Brilliance agar plates that were incubated for 48h.

Suspected colonies on VRE Brilliance, BMEG-2 and ME/CF/CX agar plates were streaked on blood agar (one isolate per morphotype). Species identification was done by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF) (Bruker Daltonik

GmbH, Bremen). Confirmed *Enterococcus* spp and *Enterobacteriaceae* spp, were tested for antibiotic susceptibility using VITEK®2 (bioMérieux) automatic system and EUCAST clinical breakpoints.

German hospital

Rectal swabs were directly plated on chromID® ESBL agar (bioMérieux) for ESBL screening and enriched Enterococcosel™ Broth (Bile Esculin Azide Broth) (BD; Becton, Dickenson and Company) was used for VRE screening and subsequently cultured on chromID® VRE agar (bioMérieux).

Species identification and antibiotic susceptibility testing was done by MALDI-TOF (Bruker Daltonik GmbH, Bremen) and VITEK®2 (bioMérieux), respectively, following EUCAST criteria. Confirmation of ESBL was performed using disk diffusion (cefotaxime 30 μg, cefotaxime 30 μg plus clavulanic acid 10 μg, ceftazidime 30 μg, ceftazidime plus clavulanic acid 10 μg, cefepime 30 μg, cefepime 30 μg plus clavulanic acid 10 μg, and cefoxitin 30 μg) (Mast Diagnostics, Derby Road, Bootle, UK).

PCRs and microarray

Enterococci isolates from The Netherlands were screened by in-house PCR for *IS16* (a marker for specific hospital associated strains), *vanA* and *vanB* genes as described previously [12, 13]. The GenoType Enterococcus (Hain Lifescience GmbH) was used in enterococci isolates from Germany, which detects species and genotypes *vanA, vanB, vanC1 and vanC2*. ESBL and VRE positive isolates were sent to our hospital for further characterization.

Enterobacteriaceae isolates resistant to third generation cephalosporins and natural chromosomal AmpC producers intermediate or resistant to cefepime were selected for DNA extraction using the UltraClean Microbial DNA Isolation Kit (MoBio, Laboratories, Inc.) and further characterized for the presence of ESBL/AmpC genes using a DNA-array (Check-MDR CT103, Check-points, Wageningen, The Netherlands) [14].

Whole-genome sequencing of VRE and ESBL-*E. coli*

Whole-genome sequencing (WGS) was performed for all ESBL-*E. coli* and VRE isolates. For each isolate, several colonies (about 5 μl) of the culture were suspended in 300 μl microbead solution, which was subjected to DNA extraction with the Ultraclean Microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA). The DNA concentration and purity were measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA,

USA) and the Qubit double-stranded DNA (dsDNA) HS and BR assay kits (Life Technologies, Carlsbad, CA, USA). One nanogram of bacterial DNA was used for library preparation. The DNA library was prepared using the Nextera XT library preparation kit with the Nextera XT v2 index kit (Illumina, San Diego, CA, USA). The library fragment length was aimed at fragments with a median size of 575 bases and was assessed with the Genomic DNA ScreenTape assay with the 2200 TapeStation system (Agilent Technologies, Waldbronn, Germany). Subsequently, the library was sequenced on a MiSeq sequencer, using the MiSeq reagent kit v2 generating 250-bp paired-end reads. Sequencing was aimed at a coverage of at least 60-fold. MiSeq data were processed with MiSeq control software v2.4.0.4 and MiSeq Reporter v2.4 (Illumina, San Diego, CA, USA). Reads were quality-trimmed using the CLC Genomics Workbench software version 9.0.1 (CLC bio, Aarhus, Denmark) using default settings except for the following modifications: "trim using quality scores was set to 0.02" and "discard reads below length was set to 15". Subsequently, trimmed-reads were *de novo* assembled with an optimal word size of 29 and a minimum contig length of 500. Metrics on raw read and assembly level are provided in Table S1.

Core genome multi locus sequence typing (cgMLST) of VRE and ESBL-*E. coli*

A genome wide gene-by-gene comparison approach was used to determine the genetic relatedness using SeqSphere+ version 3.4.0 (Ridom GmbH, Münster, Germany) [8]. Genome assemblies from the VRE isolates were analyzed using the *E. faecium* cgMLST scheme previously published, considering a cluster alert distance of 20 different alleles [10].

An *ad hoc* cgMLST and whole genome MLST (wgMLST) scheme was determined for *E. coli* isolates using the MLST+ target definer function with default parameters [15] and *Escherichia coli* K-12 as a reference (GenBank accession no. NC_010473.1). The filters applied to reference genome were: "minimum length filter" that discards genes shorter than 50 bases; "start codon filter" that discards all genes that contain no start codon at the beginning of the gene: "stop codon filter" that discards all genes that contain no stop codon, more than 1 stop codon or if the stop codon is not at the end of the gene: "homologous gene filter" that discards all genes that have fragments that occur in multiple copies in a genome (with identity ≥ 90% and more than 100 bases overlap); "gene overlap filter" that discards the shorter of two overlapping flanking genes if these genes overlap > 4 bp. The remaining genes were then used in a pairwise comparison using BLAST [8] with 45 query genomes (Table S2a). All genes of the reference genome that were common in all query genomes with a sequence identity of ≥90% and 100% overlap, and with the default parameter stop codon

percentage filter turned on, formed the final cgMLST scheme; this discards all genes that have internal stop codons in >20% of the query genomes. Additionally, 26 plasmid sequences (Table S2b) were added to exclude such genes are part of the cgMLST typing scheme. The final cgMLST scheme consisted of 1.771 targets/genes, and 2329 accessory genes were additionally included for the wgMLST scheme (Table S3 and S4). The minimum coverage of the genome assemblies was 20 times (Table S1) and the percentage of good reads included in the cgMLST were 97.6% for *E. coli* and 98.6 for *E. faecium* (Table S5 and S6).

Furthermore, to determine the genetic relatedness, the genetic distance for the *E. coli* isolates was calculated as the proportion of allele differences: dividing the number of allele differences between two genomes by the total number of genes commonly shared by those two genomes [16]. In this study thresholds for genetic distance were described to discriminate between epidemiologically related and unrelated *E. coli* isolates as 0.0095 when using wgMLST and 0.0105 for cgMLST.

E. coli STs were determined uploading genome assemblies to SeqSphere+ software following the scheme of Wirth et al [17]. Sequence genomes with no conclusive results for the 7-gene MLST were uploaded to the Enterobase database [18]. Additionally, *E. coli* major phylogenetic groups (A, B1, B2 and D) were analysed *in silico* by using MLST$^+$ Target Definer function of SeqSphere$^+$, including the *chuA*, *yjaA*, and TSPE4.C2 loci [19].

Genome assemblies were also uploaded to the Center for Genomic Epidemiology to extract information on resistance genes (ResFinder) and virulence factors (VirulenceFinder), and species confirmation for VRE and ESBL-*E.coli* (KmerFinder), and serotype (SerotypeFinder) and plasmid replicons (PlasmidFinder) for ESBL-*E.coli* [20-25].

STATISTICAL ANALYSIS

In the Dutch hospital prevalence study, associations between ESBL and ARE carriage and the following variables were analyzed: length of hospital stay, antibiotic use and (low or high risk) ward. Information was gathered by the questionnaires. Statistical analyses were performed using SPSS for Windows, v. 20.0. Univariate analyses were performed using the Fisher's exact or Chi-square methods for categorical variables. The Mann-Whitney U test was used as a non-parametric tests in variables with no normal distribution. Results with a *p*-value of ≤0.05 were considered to be statistically significant. All *p*-values are two-tailed.

RESULTS

Extended-spectrum β-lactamase (ESBL)/plasmid AmpC (pAmpC)-producing *Enterobacteriaceae*

Thirty-four isolates from 27 of the 445 included patients admitted to hospitals in the Northern Netherlands (6.1%) were confirmed ESBL and/or pAmpC positive. A total of 85.2% (23/27), 14.8% (4/27) and 3.7% (1/27) of these patients were positive for ESBL, pAmpC and both, respectively. Among the 34 isolates, 32 were *E. coli,* of which 29 were ESBL positive and three were pAmpC producers. Resistance genes detected in the *E. coli* isolates are shown in Table 2. CTX-M-15 (n=8) and CTX-M-14 (n=8) were the most prevalent ones. The other two isolates were an *E. cloacae,* containing a CTX-M-1-like gene and a pAmpC CMY-II producing *P. mirabilis.* At high risk wards, 19 patients (5.8%) were found with ESBL/pAmpC isolates compared to 8 patients (6.8%) at low risk wards (p=0.68; NS). No association was found between ESBL/pAmpC carriage and antibiotic use, length of hospital stay or ward (Table 1).

In the German hospital, a total of 18 isolates from 17 patients (17/220; 7.7%) were ESBL positive. Sixteen isolates were *E. coli* and two were *K. pneumoniae.* Of these, twelve *E. coli* and one *K. pneumoniae* isolates were available for molecular testing. Six out of twelve (50%) *E. coli* isolates and the *K. pneumoniae* isolate had a CTX-M-15 gene (Table 2).

In the retrospective Dutch community study, 11 patients (11/400; 2.75%) were ESBL/pAmpC positive: 10 ESBL *E. coli*, (CTX-M-1 being the most prevalent gene) and one pAmpC *E. coli.* (Table 2). Overall, no carbapenem resistance was observed neither in the community nor in the hospitals.

E. coli MLST and phylogenetic groups

Among ESBL/pAmpC- *E. coli* isolates from Dutch hospitals, the most prevalent STs were ST131 (clonal complex (CC) ST131; n=5, 15.6%), all of them belonging to phylogroup B2 (Table 2). In the Dutch community isolates 10 different STs were found, most of them belonging to CC ST10 (n=3, 27.3%) and one isolate to ST131 (phylogroup B2). In the German hospital, the most prevalent STs were ST38 (33.3%) and ST10 (33.3%) (Table 2).

Table 2: Molecular characterization of the *E. coli* isolates from the community and hospital patients in The Netherlands and Germany.

Sample[1]	Hospital/ Ward	β-lactamase genes	Phylogroup	ST	CC
Community					
1_Esco_CA-NL		blaCTX-M-1, blaTEM-1B	B2	131	ST131
2_Esco_CA-NL		blaSHV-12	B2	117	none
3_Esco_CA-NL		blaCMY-2	D	2309	none
4_Esco_CA-NL		blaCTX-M-1	D	57	ST350
5_Esco_CA-NL		blaCTX-M-1, blaTEM-1B	A	10	ST10
6_Esco_CA-NL		blaCTX-M-1, blaTEM-1B	B1	1079	none
7_Esco_CA-NL		blaCTX-M-1, blaTEM-1B	A	10	ST10
8_Esco_CA-NL		blaCTX-M-15	D	648	ST648
9_Esco_CA-NL		blaCTX-M-15	A	617	ST10
10_Esco_CA-NL		blaCTX-M-15	A	1312	none
11_Esco_CA-NL		blaCTX-M-14b, blaTEM-1B	D	38	ST38
Hospital					
12_Esco_HA-NL	A/ Gynaecology	blaCTX-M-15, blaTEM-1B	D	5463	none
12b_Esco_HA-NL	A/ Gynaecology	blaCTX-M-15, blaTEM-1B	D	5463	none
13_Esco_HA-NL	A/ Neurology	blaCTX-M-27	B2	131	ST131
14_Esco_HA-NL	A/ Dialysis outpatient	blaCTX-M-15, blaTEM-1B	A	93	ST168
15_Esco_HA-NL	A/ ICU	blaCMY-2, blaTEM-1B	D	354	ST354
16_Esco_HA-NL	A/ ICU	blaCTX-M-15, blaTEM-1B, blaOXA-1	B1	58	ST155
17_Esco_HA-NL	A/ ICU	blaCTX-M-15, blaTEM-1B	B1	38	ST38
18_Esco_HA-NL	A/ ICU	blaTEM-52C	B1	453	ST86
19_Esco_HA-NL	A/ ICU	blaCTX-M-1	B1	641	ST86
20_Esco_HA-NL	A/ ICU	blaSHV-12	A	5888	none
20b_Esco_HA-NL	A/ ICU	blaCTX-M-1	B1	58	ST155
21_Esco_HA-NL	B/ Gynaecology	blaCTX-M-14	B1	101	ST101
22_Esco_HA-NL	B/ Dialysis outpatient	blaCTX-M-14	B1	38	ST38
22c_Esco_HA-NL	B/ Dialysis outpatient	blaCTX-M-14	D	38	ST38
23_Esco_HA-NL	B/ Vascular surgery	blaCMY-2, blaTEM-1B	D	1508	none
24_Esco_HA-NL	B/ Neurology	blaTEM-52C	D	2064	none
25_Esco_HA-NL	B/ Neurology	blaCTX-M-3, blaTEM-1B	B2	95	ST95
25b_Esco_HA-NL	B/ Neurology	blaCTX-M-3, blaTEM-1B	D	95	ST95
26_Esco_HA-NL	C/ Gynaecology	blaCTX-M-15, blaOXA-1	B2	131	ST131
27_Esco_HA-NL	C/ Dialysis outpatient	blaCTX-M-1, blaTEM-33	A	3478	none
28_Esco_HA-NL	C/ Dialysis outpatient	blaCTX-M-14	A	10	ST10
29_Esco_HA-NL	C/ Neurology	blaCTX-M-1	B1	603	none
30_Esco_HA-NL	C/ Vascular surgery	blaCTX-M-14	A	410	ST23
31_Esco_HA-NL	D/ Vascular surgery	blaCTX-M-14, blaTEM-1B, blaOXA-1	B1	58	ST155

Sample[1]	Hospital/ Ward	β-lactamase genes	Phylogroup	ST	CC
32_Esco_HA-NL	D/ Vascular surgery	blaCTX-M-1	D	117	none
32b_Esco_HA-NL	D/ Vascular surgery	blaDHA-1, blaTEM-1B	B2	131	ST131
33_Esco_HA-NL	D/ Vascular surgery	blaCTX-M-14	D	69	ST69
33b_Esco_HA-NL	D/ Vascular surgery	blaCTX-M-14	D	69	ST69
34_Esco_HA-NL	D/ Internal medicine	blaCTX-M-55, blaOXA-1	B1	4385	none
35_Esco_HA-NL	D/ Dialysis outpatient	blaCTX-M-15, blaTEM-1B, blaOXA-1	B2	131	ST131
35b_Esco_HA-NL	D/ Dialysis outpatient	blaCTX-M-15, blaOXA-1	B2	131	ST13
36_Esco_HA-NL	D/ Dialysis outpatient	blaCTX-M-1, blaTEM-1B	B1	58	ST 155
37_Esco_HA-DE	ICU 1	blaCTX-M-15	D	38	ST38
38_Esco_HA-DE	ICU 6	blaCTX-M-14	D	38	ST38
39_Esco_HA-DE	ICU 2	blaCTX-M-14	A	10	ST10
40_Esco_HA-DE	ICU 6	blaCTX-M-15, blaTEM-1B, blaOXA-1	B1	448	ST448
41_Esco_HA-DE	Surgical ward	blaCTX-M-1, blaTEM-1B	A	10	ST10
42_Esco_HA-DE	Haemato-oncology ward	blaCTX-M-15, blaTEM-1B, blaOXA-1	A	90	ST23
43_Esco_HA-DE	ICU 4	blaCTX-M-15, blaOXA-1	A	34	ST10
44_Esco_HA-DE	ICU 3	blaTEM-187	A	10	ST10
45_Esco_HA-DE	ICU 3	blaCTX-M-15, blaOXA-1	D	38	ST38
46_Esco_HA-DE	ICU 3	blaCTX-M-1, blaTEM-1B	A	10	ST10
47_Esco_HA-DE	ICU 1	blaCTX-M-15	D	38	ST38
48_Esco_HA-DE	ICU 1	blaCTX-M-14, blaTEM-1B	D	1177	--

[1]CA: community acquired; HA: hospital acquired; NL: The Netherlands; DE: Germany; numbers refer to individual patients and a letter behind a number indicates that more than one isolate was obtained from the patient

Table 3: Variables associated with carriage of amoxicillin-resistant *E. faecium* (ARE)

Variables	ARE n=105	No ARE n=340	*p*-value*	ESBL/pAmpC n=27	No ESBL/pAmpC n=418	p-value *
Hospitalization days median (range)	12 (1-127)	3 (1-107)	$p<0.001$	4 (1-127)	4 (1-36)	$p=0.886$
Ward			$p<0.001$			$p=0.657$
– High risk (n=328)	99 (94.3%)	229 (67.4%)		19 (70.4%)	309 (73.9%)	
– Low risk (n=117)	6 (5.7%)	111 (32.6%)		8 (29.6%)	109 (26.1%)	
Antibiotic use (n=145)	62 (59%)	83 (24.4%)	$p<0.001$	7 (25.9%)	138 (33%)	$p=0.529$
– Penicillins **	26 (24.8%)	29 (8.5%)	$p<0.001$	3 (11.1%)	35 (8.4%)	$p=0.494$
– Fluoroquinolones	28 (26.7%)	15 (4.4%)	$p<0.001$	1 (3.7%)	42 (10%)	$p=0.499$
– 3rd gen cephalosporins	11 (10.5%)	19 (5.6%)	$p=0.081$	1 (3.7%)	29 (6.9%)	$p=1.00$

*Results with a *p*-value of ≤0.05 were considered to be statistically significant. All *p*-values are two-tailed. **used penicillins: benzylpenicillin, flucloxacillin, amoxicillin-clavulanic acid and piperacillin-tazobactam.

Ampicillin and vancomycin resistant *E. faecium* (ARE and VRE)

In the Dutch hospitals 105 patients (105/445; 23.6%) were colonized with ARE, including six patients (6/445; 1.3%) with VRE. All ARE were positive for *IS16* and all VRE were *vanB* positive. Colonization of ARE (and VRE) was associated with high risk wards ($p<0.001$), prolonged hospitalization ($p<0.001$) and use of antibiotics ($p=0.05$), especially penicillins and fluoroquinolones ($p<0.001$) (Table 3).

In the border German university hospital four (4/102; 3.9%) VRE isolates were isolated. Three of them were *vanA* positive and one was *vanB* positive.

In the retrospective Dutch community study, six ARE (6/400; 1.5%) were found, three of them were *IS16* positive. Only one *vanA*-VRE (1/400; 0.25%) was found, this strain was ampicillin susceptible and *IS16* negative.

cgMLST and wgMLST comparison of ESBL-*E. coli* isolates from the community and hospitals

Genome assemblies of 55 ESBL-*E. coli* (Dutch community (n=11), Dutch hospitals (n=32) and German hospital (n=12)) of this study were analyzed by a gene-by-gene approach and the allelic distance from the cgMLST and wgMLST were visualized in a minimum spanning tree (Figure 1 and Figure S1, respectively).

Six groups of isolates with a lower number of different alleles (≤ 35) by cgMLST were further analyzed. Table S7 summarizes the origin of the isolates in every group and the core and whole genome genetic distance. Those groups formed by isolates with an epidemiological link (isolated from the same patient; group 1, 4, 5a, 6a and 7), showed a core and whole genome genetic distance lower than 0.0030 and 0.0046, respectively. In addition, isolates of group 5b, although with unknown epidemiological link, had a core genetic distance of 0.0063 and a whole genome genetic distance of 0.0076. Both isolates were positive for CTX-M-14, however no plasmid replicons were found in one of them (isolate 38_Esco_HA-DE) (Table S7).

Among those groups including isolates with non (or unknown) epidemiological link, the core genome genetic distance was between 0.0122-0.0199 and the whole genome genetic distance was between 0.0104-0.0208 (groups 2, 3, 6b, and 6c; Figure 1). Resistance and virulence profiles of the isolates are shown in Table S8.

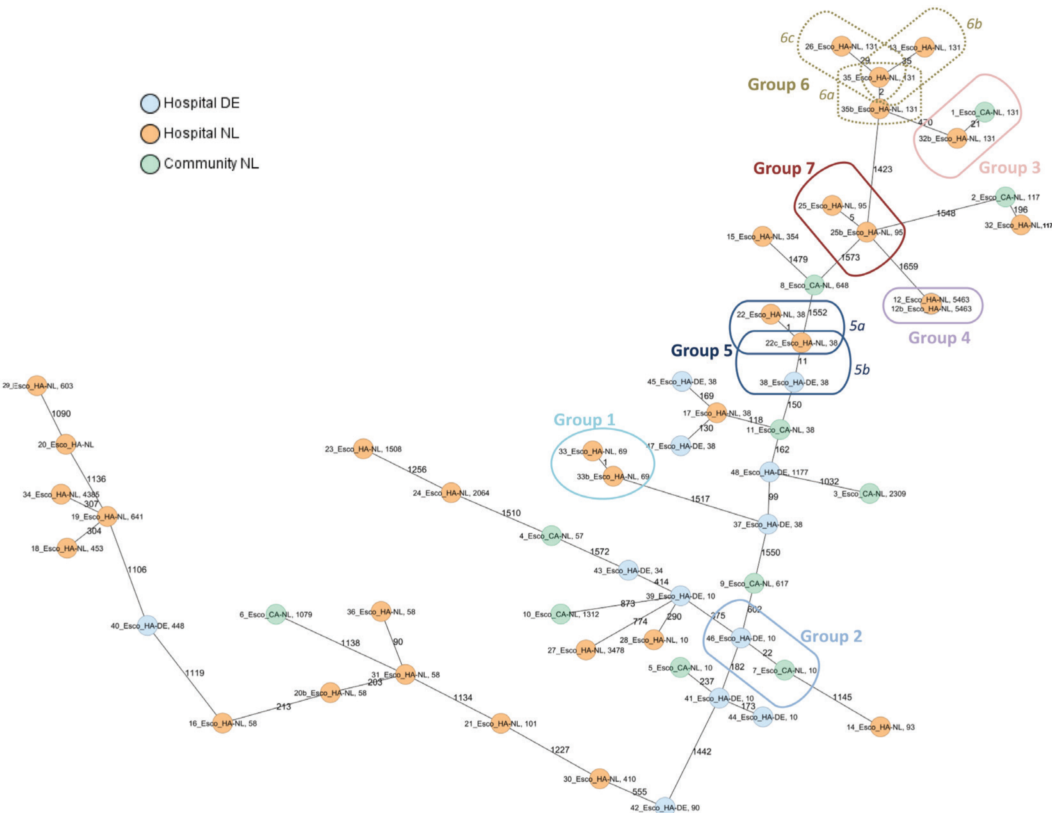
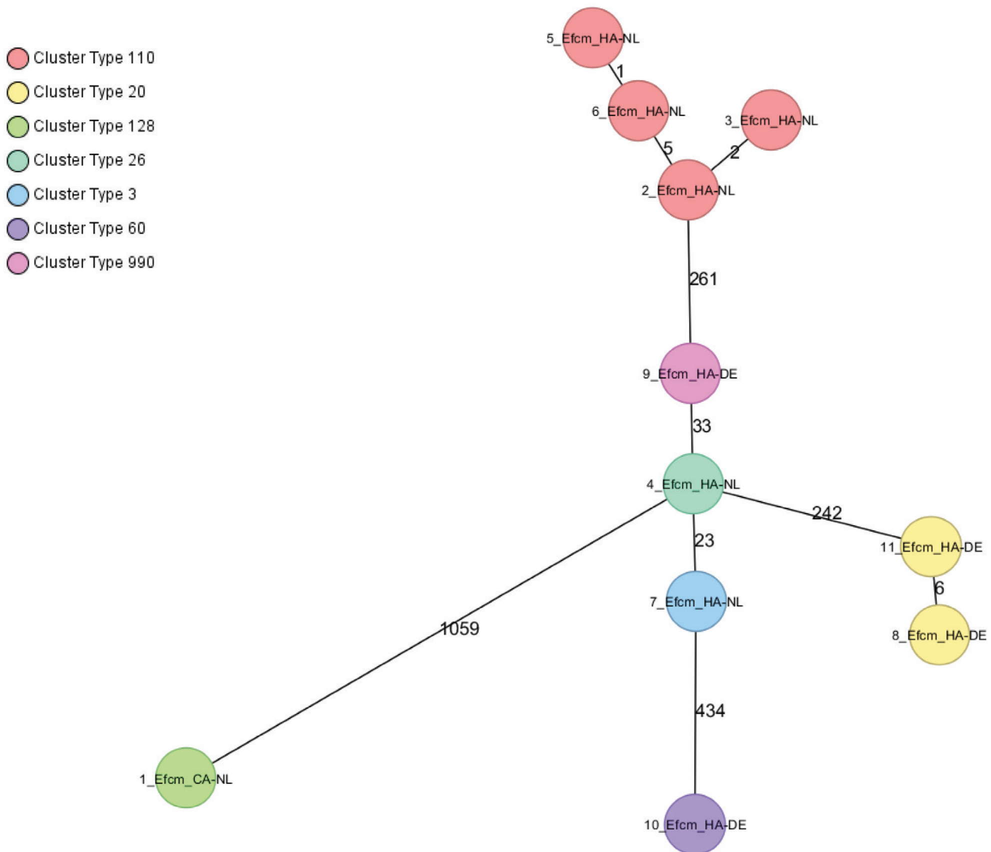


Figure 1: Minimum spanning tree of ESBL-*E. coli* isolates from hospitals and the community. Distance based on a cgMLST of 1771 genes using the parameters "pairwise ignoring missing values" during calculation. Each circle represents a genotype, colors indicate geographical origin and community or hospital. Orange: hospital-The Netherlands; blue: hospital-Germany; green: community-The Netherlands. Number of different alleles are indicated on the edges between connected isolates (nodes). The cut-off values for defining a group was 35 alleles. Isolates are presented by their ID and ST.

3

Figure 2: Minimum spanning tree of VREfcm, cgMLST based on 1423 genes using the parameters "pairwise ignoring missing values" during distance calculation. Each circle represents a genotype and colors indicate cluster types (CT). Number of different alleles are indicated on the edges between connected isolates (nodes). Isolates are presented by their ID, ST and CT.



cgMLST comparison of VRE isolates from the community and hospitals

A minimum spanning tree was created for the 11 VRE isolates (Dutch community (n=1), Dutch hospitals (n=6) and German hospital (n=4)). Two clusters of isolates from different patients were observed (Figure 2). One cluster of four *vanB*-VRE isolates from the Dutch hospital belonged to Cluster Type (CT) 110 (ST17); two isolates were from the same ward in hospital A and the other two isolates were isolated from different wards in hospital B. The other cluster of two *vanA*-VRE isolates were isolated from different wards from the German hospital (CT 20, ST203). The resistance and virulence genotypes of VRE isolates are shown in Table S8.

Nucleotide sequence accession number.

Sequence data obtained in this study has been deposited at the National Center for Biotechnology Information under BioProject no. PRJNA352198.

DISCUSSION

This study shows the molecular epidemiology of ESBL/pAmpC and HA *E. faecium* in hospitals in the Northern Dutch-German border region and the community in the Northern Netherlands. Dutch hospitals showed a prevalence for ESBL/pAmpC, VRE and ARE of 6.1%, 1.3% and 23.6% respectively, whereas the prevalence in the community was 2.75%, 0.25% and 1.5%, respectively. The German hospital had an ESBL/pAmpC prevalence of 7.7% and 3.9% for VRE.

A previous study reported a prevalence of ESBL- producing bacteria of 4.9% in the Netherlands [26], comparable to the 6.1% prevalence observed in Dutch hospitals in this study. A prevalence of 5.6% ESBL- producing *E. coli* isolates in hospitalized and ambulatory patients in Germany has been reported recently [27], which is slightly lower than the 7.7% observed in the present study.

Furthermore, we observed an ESBL- *E. coli* prevalence of 2.5% in the Northern Netherlands community, which is low compared to previous studies in other regions, in which the prevalence in the community ranged from 4.7% (2009) to 10.1% (2011) [28, 29]. This difference may have several reasons. First, ESBL prevalence may vary between regions and over time, and natural eradication of resistant *Enterobacteriaecae* might occur over time in the community [30]. Additionally, samples included in this study were only chosen from patients without any gastrointestinal complaints, a factor which otherwise has been described to be associated with high ESBL prevalence [28].

The majority of the resistance genes found in our community isolates were CTX-M-1 which is broadly disseminated among animals in Europe, especially in cattle and pigs, followed by the CTX-M-15 gene, commonly associated with human origin [27, 28]. The latter was the most frequent gene among the Dutch and German hospital isolates, in concordance with previous studies [27, 28, 31].

The pAmpC prevalence in *E. coli* in our study was 0.3%, comparable to the prevalence of 0.6% what was reported in the study of van Hoek et al. [29] (0.6% pAmpC *Enterobacteriaceae*) and somewhat lower to findings of Reuland et al. (1.3% pAmpC- *E.coli)* [32]. The most common pAmpC gene found in hospital and community isolates were CMY-II, which is together with DHA frequently found in human isolates [32].

ESBL-producing *E. coli* belonging to clonal complex ST131-phylogroup B2 are usually associated with more virulent strains [33]. These were frequently found in the Dutch hospitals included in the present study but only sporadically in the community samples. This CC ST131-phylogroup B2 was also prevalent in a study carried out in hospitals in the Rotterdam region [34]. CC ST10 was predominant among the ESBL- producing *E. coli* in the community, the same clonal complex was also described to be prevalent in another Dutch study in community patients [28].

We observed an overall ARE and VRE prevalence in hospitalized patients of 23.6% and 1.3%, respectively. Similar observations were made in a study performed in Dutch hospitals in 2008 reporting ARE carriage rates of 10-16% upon admission and 15-39% on acquisition in haematology and gastroenterology/nephrology wards [35]. The clinical significance of enterococcal infections and active VRE screening has been a matter of discussion. However, in immunocompromised patients, high morbidity and mortality rates have been reported in infections caused by enterococci [36] . In this study ARE/VRE carriage was associated with prolonged hospitalization and antibiotic use, which is in line with previous literature [37]. We found a high carriage rate of ARE in high risk wards (30.2%). Notably, these patients may be at risk for a subsequent infection. Since 2011, VRE started to become a problem in multiple hospitals in the Netherlands: a total of fourteen hospitals were affected with outbreaks of VRE in October 2012 [38]. However, in this study a prevalence of VRE (*vanB*) carriage of only 1.3% was found. This is probably due to extensive infection prevention measures and successful outbreak management control. The prevalence of 1.3% is similar to what has been previously published in the Netherlands, with prevalence rates ranging from 1.4%- 2% in the 90s [39, 40]. The VRE prevalence in the German hospital was slightly higher (3.9%),

though it is known that Germany has a higher VRE prevalence compared to the Netherlands [41].

In our Dutch community one *vanA*-VRE was found, that was ampicillin susceptible and *IS16* negative, indicative for a non-hospital origin [4, 5]. Endtz *et al.* reported a higher number of VRE in the community (2%), however this study did not include information about ampicillin resistance nor *IS16* which makes it difficult to determine if they had a hospital or non-hospital origin [4, 5].

The cgMLST analysis in our study showed heterogeneity among *E. coli* species, and isolates were genetically distributed independently of their origin. The hospital and community ESBL- *E. coli* isolates included in this study did not show any genetic relatedness except for the ones isolated from the same patient and for two isolates (group 5b) from patients in different hospitals across the Dutch-German border, in a distance of approximately 200km and with no known epidemiological link. The patient from the Dutch hospital was a dialyses outpatient (isolation date December 2012) whereas the patient from the German hospital was admitted to ICU (isolation date November 2012). Interestingly, both isolates harbored the same ESBL gene and virulence factors.

Genetic relatedness was found between four VRE isolates (CT110) from patients from two different Dutch hospitals (Figure 2), which indicates transmission between wards, but also between hospitals in a close geographical region similar to findings of a previous population-based study of VRE using WGS that also showed intra- and inter-regional spread of closely related VRE isolates [42]. Although no genetic relatedness was found between VRE isolates of Dutch and German hospitals, the numbers of VRE isolates were too low to draw definite conclusions. It is known that several VRE cluster types co-circulate in Germany and the Netherlands (data not shown). However, only some laboratories have implemented the use of cgMLST in their routine to analyse VRE outbreaks and more epidemiological studies are needed to investigate cross-border transmission of VRE.

To our knowledge there are no similar studies that compare and investigate the molecular epidemiology of ESBL *E.coli* and VRE in hospitals and the community by WGS. Recently, the same approach has been used to study the clonality of ESBL- producing *Enterobacteriaceae* from environmental and stool samples from farmers suggesting possible cross-transmission between the farmers and the environment. This was only based on number of allele differences [16, 43] which makes it difficult to interpret results without considering the total number of genes included in the cgMLST scheme. In our study, we determined the genetic relatedness between ESBL-*E. coli* using cgMLST or wgMLST comparison and genetic

distance calculation. These results were in concordance with the genetic distance thresholds of 0.0095 (wgMLST) and 0.0105 (cgMLST) previously established for *E. coli* based on known existing epidemiological links by analysing more than 2.000 ESBL-*Enterobacteriaceae* isolates from Dutch hospitals [16].

In another study, a cgMLST approach for several MDR bacteria was prospectively used for taking relevant infection control decisions in a hospital setting [44]. A threshold of >10 differing alleles was defined to exclude nosocomial transmission of MDR *E. coli* [44]. If we would have applied this threshold we would have missed the genetic relatedness between isolates belonging to group 5b, presenting 11 different alleles (Figure 1 and Table S7). This highlights that thresholds based on number of allele differences are only applicable to specific collections within a study, whereas the genetic distance calculation seems to give a more objective result, independently of the analysed population.

We acknowledge this study has some limitations. No community study in the German cross-border region, neither ARE monitoring in the German hospital were performed. Laboratory methods for isolation of ESBL *Enterobacteriaceae* and VRE differed between Dutch and German hospitals since no enrichment broth was used in Germany, however selective media agar was used in both regions. Since this study was anonymous, some epidemiological data were not available which makes it more difficult to draw conclusions regarding genetic relatedness among isolates between patients.

In conclusion, the results of this study suggest that ESBL/pAmpC-*E.coli* circulate in the hospital and the community, although a higher prevalence of ESBL/pAmpC-*E. coli* was observed in hospitals compared to the community in the Northern Netherlands. Hospitals in the Netherlands and Germany showed a similar prevalence of ESBL/pAmpC-*Enterobacteriaceae.* VRE prevalence was still low in the hospital as well as in the community in the Northern Netherlands. The German hospital showed a slightly higher VRE prevalence compared to hospitals in the Northern Netherlands. Nosocomial but no cross-border transmission of VRE was observed in this study. Epidemiologically related ESBL-*E. coli* and VRE were uncommon across the Dutch-German border in the studied population, as only two ESBL- *E. coli* isolates from a Dutch and a German hospital were genetically similar. Cooperation between bordering countries and continuous monitoring using high discriminatory typing methods are still necessary to keep the epidemiology of resistant pathogens updated thereby helping to control their spread.

These results were partially presented at the ECCMID conference 2016, Amsterdam.

Acknowledgements

We would like to thank Dr. C. R.C. Doorenbos, M. Zigterman, W. Postma, P. Rurenga, N. Welles, A. Woudstra, S. de Vries, and A. J. Stellingwerf for their participation in this study.

Funding

This study was supported by the Interreg IVa-funded projects EurSafety Heatlh-net (III-1-02=73) and SafeGuard (III-2-03=025), part of a Dutch-German cross-border network supported by the European Commission, the German Federal States of Nordrhein-Westfalen and Niedersachsen, and the Dutch provinces of Overijssel, Gelderland, and Limburg.

Transparency declarations

None to declare

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Table S1: metrics raw data and assemblies

	Number of contigs <1000	N50 >15.000	Max contig length >50.000	Contig total bp	Coverage >30x	% reads used >90%	% of expected genome size >90% - <115%	Reads count	Reads average length	Count matched
E. coli										
1_Esco_CA-NL	118	191682	358090	5355780	84,94	99,60	98,5	2021636	225,03	2013594
2_Esco_CA-NL	108	163016	364102	5283863	86,38	99,44	97,1	2721752	167,69	2706482
3_Esco_CA-NL	247	99795	322831	5808942	55,29	99,11	106,8	1648839	194,8	1634095
4_Esco_CA-NL	141	98292	267208	5427644	76,43	99,59	99,8	1841350	225,3	1833826
5_Esco_CA-NL	166	76293	203186	4967893	73,15	99,45	91,3	1624869	223,65	1615992
6_Esco_CA-NL	65	193003	705749	4964881	90,86	99,48	91,3	2265390	199,13	2253707
7_Esco_CA-NL	107	125308	478078	4861038	91,40	99,53	89,4	1986025	223,72	1976643
8_Esco_CA-NL	126	135970	505790	5245727	64,62	99,28	96,4	1532958	221,12	1521955
9_Esco_CA-NL	107	126118	355226	4742141	134,39	99,59	87,2	3849660	165,54	3833954
10_Esco_CA-NL	131	68898	215278	4592628	86,85	99,59	84,4	1751629	227,72	1744519
11_Esco_CA-NL	119	148112	373540	5351130	65,41	99,62	98,4	1536467	227,81	1530698
12_Esco_HA-NL	55	223440	572354	4492147	166,69	99,20	82,6	5836391	128,3	5789524
12b_Esco_HA-NL	75	127820	354607	4496772	35,51	99,54	82,7	938899	170,08	934610
13_Esco_HA-NL	86	191392	570686	5038933	100,13	99,34	92,6	2758668	182,9	2740565
14_Esco_HA-NL	175	150214	284655	4832324	93,65	98,84	88,8	3537371	127,93	3496430
15_Esco_HA-NL	138	190832	408059	5347576	97,36	99,39	98,3	3296572	157,93	3276511
16_Esco_HA-NL	119	172411	420618	5194891	84,34	99,55	95,5	3354184	130,63	3339148
17_Esco_HA-NL	116	166159	602705	5399155	127,76	99,50	99,2	4039881	170,74	4019870
18_Esco_HA-NL	137	145507	275025	5215799	52,55	99,22	95,9	1799021	152,35	1784973
19_Esco_HA-NL	105	128899	313660	4866096	111,72	99,59	89,5	3272321	166,14	3258844
20_Esco_HA-NL	132	109269	363528	5099690	84,79	99,34	93,7	2820330	153,31	2801679

	Number of contigs <1000	N50 >15.000	Max contig length >50.000	Contig total bp	Coverage >30x	% reads used >90%	% of expected genome size >90% - <115%	Reads count	Reads average length	Count matched
20b_Esco_HA-NL	86	217811	343117	4857676	41,45	99,58	89,3	1169242	172,21	1164342
21_Esco_HA-NL	136	111742	305200	5279160	75,98	99,57	97,0	1825862	219,69	1818071
22_Esco_HA-NL	89	147942	294513	5142005	86,44	99,62	94,5	2166676	205,15	2158499
22c_Esco_HA-NL	203	62928	186153	5127236	29,91	99,35	94,3	970810	157,98	964475
23_Esco_HA-NL	87	133573	352442	4976051	87,83	99,69	91,5	1900596	229,94	1894797
24_Esco_HA-NL	114	109880	235251	4819129	67,12	99,53	88,6	1411446	229,18	1404817
25_Esco_HA-NL	109	153918	442015	5261580	61,65	98,52	96,7	1463690	221,63	1442025
25b_Esco_HA-NL	458	22197	110084	5244141	20,48	98,84	96,4	656398	163,65	648756
26_Esco_HA-NL	89	174102	404229	5116427	87,50	99,71	94,1	1964945	227,83	1959317
27_Esco_HA-NL	214	65691	247479	5196831	85,17	99,51	95,5	1946210	227,43	1936665
28_Esco_HA-NL	56	173305	457591	4784220	76,49	99,66	87,9	1595930	229,3	1590447
29_Esco_HA-NL	205	89142	203044	5187114	58,84	99,61	95,4	1325362	230,28	1320175
30_Esco_HA-NL	82	148216	451007	4971572	83,66	99,51	91,4	1848393	225,02	1839374
31_Esco_HA-NL	158	83469	233486	5011249	82,42	99,68	92,1	1817965	227,2	1812137
32_Esco_HA-NL	109	153918	442015	5261580	61,65	98,52	96,7	1463690	221,63	1442025
32b_Esco_HA-NL	189	51700	148831	5078255	22,92	99,32	93,4	704857	165,15	700084
33_Esco_HA-NL	58	244539	407504	4995036	117,53	99,66	91,8	3375428	173,92	3364104
33b_Esco_HA-NL	89	253460	414095	4993860	49,76	99,62	91,8	1613400	154,02	1607303
34_Esco_HA-NL	139	112993	295864	5152838	89,12	99,62	94,7	2031654	226,04	2023889
35_Esco_HA-NL	124	119161	359876	5059658	67,81	99,06	93,0	1577416	217,52	1562551
35b_Esco_HA-NL	235	56790	187134	4989725	22,75	99,28	91,7	633934	179,08	629393
36_Esco_HA-NL	132	131356	402574	5095672	71,40	99,52	93,7	2224309	163,57	2213674
37_Esco_HA-DE	108	133196	497892	5370809	84,49	99,70	98,7	1872235	242,36	1866657

	Number of contigs <1000	N50 >15.000	Max contig length >50.000	Contig total bp	Coverage >30x	% reads used >90%	% of expected genome size >90% - <115%	Reads count	Reads average length	Count matched
38_Esco_HA-DE	61	274210	838823	5526501	40,34	99,19	101,6	994920	224,05	986857
39_Esco_HA-DE	44	210315	481462	4692670	76,91	99,64	86,3	1525985	236,52	1520565
40_Esco_HA-DE	114	112692	287503	4908468	42,50	99,45	90,2	861779	242,07	857028
41_Esco_HA-DE	92	140654	439539	4832312	64,40	99,68	88,8	1297992	239,76	1293844
42_Esco_HA-DE	82	274276	718677	4974466	66,48	99,72	91,4	1397607	236,61	1393625
43_Esco_HA-DE	193	70632	152492	4956801	55,29	99,22	91,1	1144482	239,47	1135568
44_Esco_HA-DE	139	112193	287998	5019313	68,54	99,21	92,3	1459096	235,77	1447579
45_Esco_HA-DE	212	66407	203445	5365665	57,30	99,32	98,6	1344500	228,69	1335308
46_Esco_HA-DE	82	218246	358823	4973327	61,95	99,57	91,4	1301009	236,83	1295377
47_Esco_HA-DE	143	119425	279283	5285998	64,06	99,66	97,2	1434793	235,99	1429863
48_Esco_HA-DE	190	75705	319715	5421044	70,00	98,90	99,7	1628210	233,07	1610227
E. faecium										
1_Efcm_CA-NL	99	69218	233412	2528636	101,03	98,97	86,3	1085629	235,31	1074425
2_Efcm_HA-NL	175	36317	148624	2991184	126,05	99,34	102,1	1588025	237,42	1577537
3_Efcm_HA-NL	182	35172	148166	2994909	156,94	99,33	102,2	2019371	232,76	2005812
4_Efcm_HA-NL	178	34655	146554	2941833	130,21	99,44	100,4	1638752	233,75	1629644
5_Efcm_HA-NL	191	31780	111795	2942805	161,66	98,83	100,4	2068528	229,98	2044374
6_Efcm_HA-NL	191	32824	106547	2973627	138,89	98,82	101,5	1770177	233,32	1749347
7_Efcm_HA-NL	142	47512	146738	2884089	143,15	99,60	98,4	1588545	259,89	1582145
8_Efcm_HA-DE	178	34271	134529	2982597	136,10	99,52	101,8	1615214	251,31	1607512
9_Efcm_HA-DE	167	46221	145187	2955402	150,25	99,59	100,9	1756628	252,79	1749495
10_Efcm_HA-DE	221	36608	190812	2987218	169,03	99,58	102,0	1980600	254,94	1972316
11_Efcm_HA-DE	175	37291	134580	3063945	107,29	99,19	104,6	1449009	226,86	1437252

Table S2a: Finished *E.coli* query genomes used in this study to develop and *ad hoc* cgMLST scheme (n=45). One representative isolate of every ST from every collection (community NL (n=10), Dutch hospitals (n=20) and German hospital (n=6) of the present study and 9 *E. coli* genomes from Dutch patients and farmers previously published (de Been et al. 2014)

Strain	Source	Place of isolation	BioSample. No.	Ref.
148	Human (blood)	Utrecht	SAMN02471499	De Been *et al*
320	Human (urine)	Utrecht	SAMN02471480	De Been *et al*
1350	Human (urine)	Leeuwarden	SAMN02471497	De Been *et al*
1365	Human (urine)	Leeuwarden	SAMN02471498	De Been *et al*
597	Human (urine)	Groningen	SAMN02471510	De Been *et al*
606	Human (pulmonary)	Groningen	SAMN02471485	De Been *et al*
FAH1	Human (faeces)	farm A	SAMN02471475	De Been *et al*
FBH1	Human (faeces)	farm B	SAMN02471517	De Been *et al*
FCH1	Human (faeces)	farm	SAMN02471511	De Been *et al*
1_Esco_CA-NL	Human	Community - NL	SAMN05967539	This study
2_Esco_CA-NL	Human	Community - NL	SAMN05977321	This study
3_Esco_CA-NL	Human	Community - NL	SAMN05977322	This study
4_Esco_CA-NL	Human	Community - NL	SAMN05977323	This study
5_Esco_CA-NL	Human	Community - NL	SAMN05977324	This study
6_Esco_CA-NL	Human	Community - NL	SAMN05977325	This study
8_Esco_CA-NL	Human	Community - NL	SAMN05977327	This study
9_Esco_CA-NL	Human	Community - NL	SAMN05977328	This study
10_Esco_CA-NL	Human	Community - NL	SAMN05977329	This study
11_Esco_CA-NL	Human	Community - NL	SAMN05977330	This study
12_Esco_HA-NL	Human	Hospital - NL	SAMN05977331	This study
13_Esco_HA-NL	Human	Hospital - NL	SAMN05977333	This study
14_Esco_HA-NL	Human	Hospital - NL	SAMN05977334	This study
15_Esco_HA-NL	Human	Hospital - NL	SAMN05977335	This study
16_Esco_HA-NL	Human	Hospital - NL	SAMN05977336	This study
17_Esco_HA-NL	Human	Hospital - NL	SAMN05977337	This study
18_Esco_HA-NL	Human	Hospital - NL	SAMN05977338	This study
19_Esco_HA-NL	Human	Hospital - NL	SAMN05977339	This study
20_Esco_HA-NL	Human	Hospital - NL	SAMN05977340	This study
21_Esco_HA-NL	Human	Hospital - NL	SAMN05977342	This study
23_Esco_HA-NL	Human	Hospital - NL	SAMN05977345	This study
24_Esco_HA-NL	Human	Hospital - NL	SAMN05977346	This study
25_Esco_HA-NL	Human	Hospital - NL	SAMN05977347	This study
27_Esco_HA-NL	Human	Hospital - NL	SAMN05977350	This study
28_Esco_HA-NL	Human	Hospital - NL	SAMN05977351	This study
29_Esco_HA-NL	Human	Hospital - NL	SAMN05977352	This study
30_Esco_HA-NL	Human	Hospital - NL	SAMN05977353	This study
32_Esco_HA-NL	Human	Hospital - NL	SAMN05977355	This study
33_Esco_HA-NL	Human	Hospital - NL	SAMN05977357	This study
34_Esco_HA-NL	Human	Hospital - NL	SAMN05977359	This study

Strain	Source	Place of isolation	BioSample. No.	Ref.
37_Esco_HA-DE	Human	Hospital - DE	SAMN05977363	This study
39_Esco_HA-DE	Human	Hospital - DE	SAMN05977365	This study
40_Esco_HA-DE	Human	Hospital - DE	SAMN05977366	This study
41_Esco_HA-DE	Human	Hospital - DE	SAMN05977367	This study
42_Esco_HA-DE	Human	Hospital - DE	SAMN05977368	This study
43_Esco_HA-DE	Human	Hospital - DE	SAMN05977369	This study

de Been, M., V. F. Lanza, M. de Toro, J. Scharringa, W. Dohmen, Y. Du, J. Hu, et al. 2014. Dissemination of cephalosporin resistance genes between escherichia coli strains from farm animals and humans by specific plasmid lineages. PLoS Genetics 10 (12) (Dec 18): e1004776.

Table S2b: Finished *plasmid* genomes for exclusion of genes with BLAST matches >90% and >100bp length found within the query sequences used in this study to develop a cgMLST scheme.

Strain	Plasmid	GenBank Acc. No.
Escherichia coli 042	pAA	NC_017627.1
Escherichia coli APEC O1	pAPEC-O1-R	NC_009838.1
Escherichia coli ETEC H10407	p948	NC_017724.1
Escherichia coli JJ1886	pJJ1886_5	NC_022651.1
Escherichia coli O104:H4 str. 2009EL-2050	p09EL50	NC_018651.1
Escherichia coli O104:H4 str. 2011C-3493	pESBL-EA11	NC_018659.1
Escherichia coli O111:H- str. 11128	pO111_1	NC_013365.1
Escherichia coli O127:H6 str. E2348/69	pE2348-2	NC_011602.1
Escherichia coli O157:H7 EDL933	pO157	NC_007414.1
Escherichia coli O157:H7 str. TW14359	pO157	NC_013010.1
Escherichia coli O157:H7 str. Sakai	pO157	NC_002128.1
Escherichia coli O26:H11 str. 11368	pO26_1	NC_013369.1
Escherichia coli O55:H7 str. CB9615	pO55	NC_013942.1
Escherichia coli O55:H7 str. RM12579	p12579_1	NC_017653.1
Escherichia coli O7:K1 str. CE10	pCE10A	NC_017647.1
Escherichia coli O83:H1 str. NRG 857C	pO83_CORR	NC_017659.1
Escherichia coli PMV-1	pHUSEC411like	NC_022371.1
Escherichia coli SE11	pSE11-1	NC_011419.1
Escherichia coli SE15	pECSF1	NC_013655.1
Escherichia coli UM146	pUM146	NC_017630.1
Escherichia coli UMN026	p1ESCUM	NC_011749.1
Escherichia coli UMNK88	pUMNK88	NC_017645.1
Escherichia coli UTI89	pUTI89	NC_007941.1
Escherichia coli W	pRK1	NC_017637.1
Escherichia coli W	pRK1	NC_017665.1
Escherichia coli Xuzhou21	pO157	NC_017907.1

Table S3: *E. coli* cgMLST 1771 targets.
Available online: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01914/full#supplementary-material

Table S4: Accessory genes included in the wgMLST scheme of *E. coli*.

Available online: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01914/full#supplementary-material

Table S5: E. coli cgMLST allele types for distance calculation and percentage of good targets/genes.

Available online: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01914/full#supplementary-material

Table S6: *E. faecium* cgMLST allele types for distance calculation and percentage of good targets/genes.

Available online: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01914/full#supplementary-material

Table S7: Genetic distance for pairwise comparisons of grouped ESBL- *E. coli* isolates.

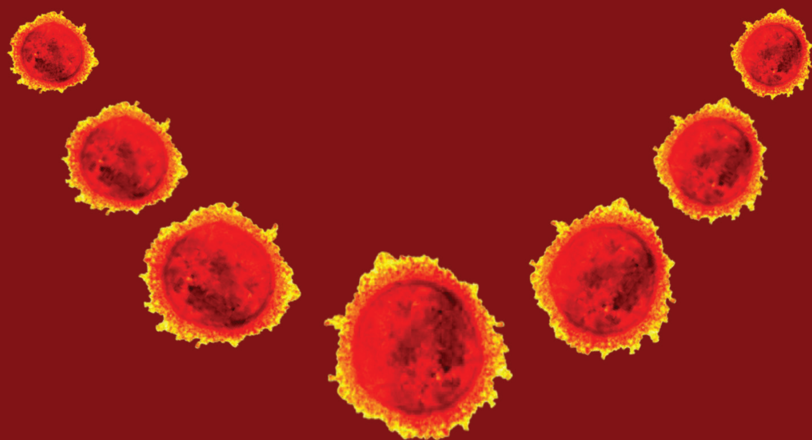
						genetic distance	
Sample ID	**ST**	**Phylogroup**	**Origin**	**Ward**	**Groups**	**cgMLST**	**wgMLST**
33_Esco_HA-NL	69	D	HA-NL	Vascular surgery	group 1	0,0006	0,0008
33b_Esco_HA-NL	69	D	HA-NL	Vascular surgery			
7_Esco_CA-NL	10	A	CA-NL	-	group 2	0,0124	0,0135
46_Esco_HA-DE	10	A	HA-DE	ICU			
1_Esco_CA-NL	131	B2	CA-NL	-	group 3	0,0122	0,0104
32b_Esco_HA-NL	131	B2	HA-NL	Vascular surgery			
12_Esco_HA-NL	5463	D	HA-NL	Gynaecology	group 4	0	0,0004
12b_Esco_HA-NL	5463	D	HA-NL	Gynaecology			
22_Esco_HA-NL	38	B1	HA-NL	Dialysis outpatient	group 5a	0,0006	0,0008
22c_Esco_HA-NL	38	D	HA-NL	Dialysis outpatient	group 5a /5b		
38_Esco_HA-DE	38	D	HA-DE		group 5b	0,0063	0,0076
35b_Esco_HA-NL	131	B2	HA-NL	Dialysis outpatient	group 6a	0,0012	0,0009
35_Esco_HA-NL	131	B2	HA-NL	Dialysis outpatient	group 6a / 6b / 6c		
13_Esco_HA-NL	131	B2	HA-NL	Neurology	group 6b	0,0199	0,0208
26_Esco_HA-NL	131	B2	HA-NL	Gynaecology	group 6c	0,0165	0,0170
25_Esco_HA-NL	95	B2	HA-NL	Neurology	group 7	0,0030	0,0046
25b_Esco_HA-NL	95	B2	HA-NL	Neurology			

HA: hospital acquired; CA: community acquired; NL: The Netherlands; DE: Germany

Table S8: Results of ResFinder, VirulenceFinder, PlasmidFinder, and SerotypeFinder for *E. coli* and *E. faecium* isolates.
Available online: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01914/full#supplementary-material

Figure S1: Minimum spanning tree of ESBL-*E. coli* isolates from hospitals and the community. Distance based on a wgMLST of 4100 genes (cgMLST of 1771 genes and 2329 accessory genes) using the parameters "pairwise ignoring missing values" during calculation. Each circle represents a genotype, colors indicate geographical origin and community or hospital. Orange: hospital-The Netherlands; blue: hospital-Germany; green: community-The Netherlands. Number of different alleles are indicated on the edges between connected isolates (nodes). The same groups considered by cgMLST analysis are highlighted. Isolates are presented by their ID and ST.

Hospital DE
Hospital NL
Community NL



Algorithm for pre-emptive glycopeptide treatment in patients with haematologic malignancies and an *Enterococcus faecium* bloodstream infection

4

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Keywords: *Enterococcus faecium*, haematologic patients, risk factors, glycopeptides, antibiotic stewardship

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ABSTRACT

Introduction:

Nowadays *Enterococcus faecium* has become one of the most emerging and challenging nosocomial pathogens. The aim of this study was to determine risk factors in haematology patients who are at risk of an *Enterococcus faecium* bloodstream infection (BSI) and should be considered for pre-emptive glycopeptide treatment. With these identified risk factors a prediction model can be developed for clinical use.

Methods:

Retrospectively clinical and microbiological data in 33 patients with an *E. faecium* BSI were compared to 66 control patients during a 5-year period at the haematology ward. Multivariate logistic regression was used to explore the independent risk factors and a prediction model was developed to determine the risk of an *E. faecium* BSI.

Results:

E. faecium BSIs were found to be associated with high mortality rates. Independent risk factors for *E. faecium* BSI were colonization with *E. faecium* 30 days prior to blood culture (OR 5.71; CI 1.7-18.7), combination of neutropenia and abdominal focus (4.37; 1.4-13.4), age > 58 years (4.01; 1.3-12.5), hospital stay prior to blood culture > 14 days (3.55; 0.98-12.9) and CRP (C-reactive protein) level >125mg/L (4.37; 1.1-10.2).

Conclusion:

Using data from this study, risk stratification for the development of an *E. faecium* BSI in patients with haematological malignancies is possible. Pre-emptive treatment should be considered in those patients who are at high risk. Using a prediction model as designed in this study, antibiotic stewardship in terms of prudent use of glycopeptides can be improved and might be helpful in controlling further spread of VRE.

INTRODUCTION

Enterococcus faecium has become one of the most important, emerging and challenging nosocomial pathogens [1]. It is a difficult to treat pathogen due to intrinsic resistances to cephalosporins, aminoglycosides (low-level resistance), clindamycin and trimethoprim-sulfamethoxazole [2]. Moreover, it has the ability to easily acquire virulence or antibiotic resistance genes trough transfer of plasmids, chromosomal exchange or mutation [3].

Due to the resistance of multiple antibiotics, the treatment of choice in serious *E. faecium* infections is glycopeptides. However, prudent use of vancomycin is needed as it is associated with an increased risk for VRE infection and colonization [4]. The emergence of VRE has been reported one to two decades ago in the United States [5]; more recently alarming reports are now coming from many countries in Europe [6].

Several studies have pointed out the existence of two subpopulations of *E. faecium*: commensal/community-associated (CA) strains and clinical or hospital associated (HA) strains, whereas the latter is also referred as the clonal complex 17 (CC-17) group [7]. These HA/CC-17 strains are associated with ampicillin resistance; the rise and replacement of *E. faecium* as the predominant enterococcus species are especially due to these strains [8].

A predominant part of the nosocomial *E. faecium* bloodstream infections concerns patients with haematologic malignances who are immunocompromised by their severe disease and intensive treatment. Whereas it often is debated whether to treat *E. faecium* as a real pathogen, several studies have shown high morbidity and mortality rates for enterococcal bacteremia (mortality rates ranging from 25% to 51%), especially in immunocompromised patients [9-11]. Moreover, the mortality rates increases with inappropriate antimicrobial therapy [12].

After coagulase negative staphylococci (CoNS), streptococci and *Escherichia coli* (*E. coli*), *E. faecium* is the most predominant species isolated among blood cultures at the haematology unit of our hospital. Compared to other pathogens such as CoNS, *E. coli, Pseudomonas aeruginosa (P. aeruginosa)* and streptococci which remained stable or decreased, *E. faecium* increased for the periods 1998-2006 (3.1%) and 2007-2010 (12.8%) which is 4.1 times more.

Since patients with haematologic malignancies are highly prone to infection, prophylactic antibiotics are used to prevent and reduce any risk of infection. In our haematology ward penicillin and ciprofloxacin or co-trimoxazol or colistine or tobramycin (orally) are used depending on the resistance pattern of bacteria found in surveillance cultures. In case a haematology patient presents with neutropenic fever or other clinical signs of infection,

blood cultures are taken and empirical broad-spectrum antibiotic treatment is started, which is piperacillin-tazobactam.

Glycopeptides are not recommended as a standard part of the initial antibiotic regimen for fever and neutropenia. Moreover, as noted earlier, for the further prevention and control of VRE it is necessary to control the use of glycopeptide antibiotics. At this moment, glycopeptides are only added in case of a positive blood culture with *E. faecium* or oxacillin resistant CoNS. However blood culture results and their susceptibilities are only available after one or more days after blood samples are drawn.

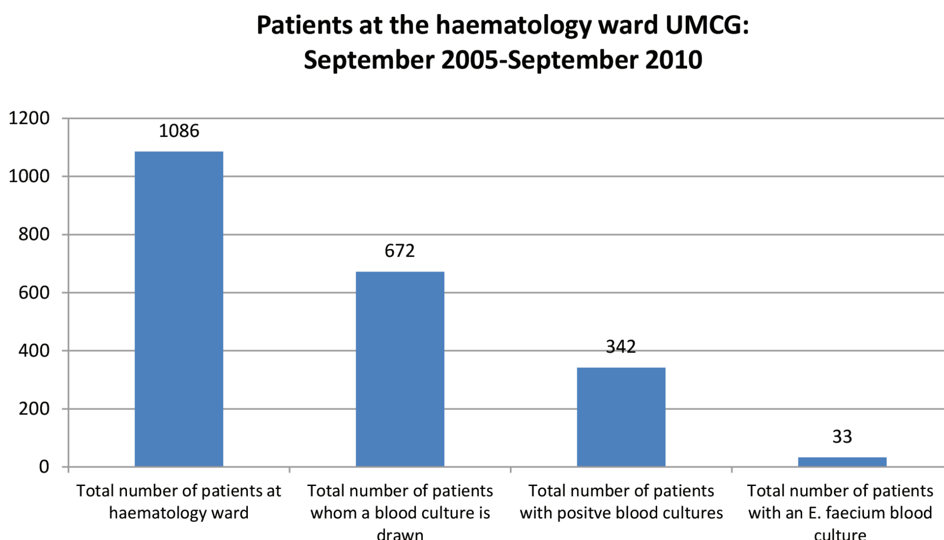
Therefore the aim of this study is to identify possible risk factors in those haematology patients who are at high risk of *E. faecium* bloodstream infection in order to develop a prediction model for clinical stringent use. This can be useful in the decision of pre-emptive therapy with glycopeptides together with the initial empirical antibiotic treatment at the moment a blood culture is taken.

METHODS

Study design and population

The University Medical Center Groningen (UMCG) is a 1300-bed tertiary center and has a 27-bed haematology ward. This ward has four 4 patient rooms, one double room and nine private rooms. Patients were identified by a search of the laboratory electronic database for all blood cultures between September 2005 and September 2010 from the haematology ward. In this period a total of 1086 patients were admitted to the haematology ward of whom 672 blood cultures were taken. (Figure 1) Case patients were identified by a search for all blood cultures positive for E. faecium. Of each patient with an E. faecium blood culture, the first positive blood culture was selected: a total of 33 patients with E. faecium blood cultures were identified. For the main purpose of our study, (an algorithm to decide whether or not to add glycopeptides to the initial empirical antibiotic therapy at the moment a blood culture is taken) we choose to use a selection of all the patients of which a blood culture was taken (positive as well as negative), except those with *E. faecium* blood culture (n=672-33=639). After all, this whole group had the same grounds to obtain a blood culture at the (retrospective) moment the blood culture was drawn. This would also be the case in prospective situations where this algorithm could be applied on. A total of 66 control patients were randomly selected: first a patient was randomly selected; subsequently a blood culture was randomly selected. Patients were not matched for age or sex.

Figure 1. Patients at the haematology ward of the UMCG during the period September 2005-September 2010: Thirty-three of the patients with positive blood cultures (672) had an *E. faecium* blood culture (~5%)



Data collection

Patient data were gathered by reviewing hospital electronic records and stored hard-copy records. The date the blood culture was taken was chosen as day 0 and from that point all data were reviewed all data retrospectively. Clinical data collected included information of underlying disease, admission status, co-morbidities, neutropenia, C-reactive protein (CRP) levels, fever and signs of organ failure prior to blood culture. Microbiological data collected included clinical source of infection, information about *E. faecium* colonization and antibiotic use 30 days prior to positive blood culture. If a patient had diarrhea, records were also reviewed for *Clostridium difficile*. Antibiotic susceptibility patterns, presence of polymicrobial bacteremia and positive galactomannan tests were gathered. Antibiotic treatment with vancomycin or teicoplanin for *E. faecium* bacteremia was evaluated. Outcomes were measured by need of ICU admission and mortality at 7 and 30 days after blood culture.

Clinical notifications and definitions

During the retrospective study period, blood cultures were drawn for neutropenic fever or other clinical signs for infection. Fever was defined as temperature >38.5 °C or > 38 °C for 24 hours was a reason for further examination. An absolute neutrophil count below 0.5 x 10^9/L was defined as neutropenia. For organ failure the following definitions were used: renal failure was defined as creatinin >176μmol/L, hepatic failure as bilirubin >43mmol/L and pulmonary failure as bilateral

lung infiltrates or signs of acute respiratory distress syndrome (ARDS). These definitions were according to guidelines used for defining organ failure in severe sepsis [13]. Polymicrobial infection was defined as a micro-organism other than *E. faecium* within ± 7 days of the blood culture. For the controls it was defined as an additional micro-organism within ± 7 days of a positive blood culture. In this definition less pathogenic micro-organisms such as CoNS, *Corynebacteriae*, *Micrococcus* spp. and *Bacillus* spp. as an additional micro-organism were excluded.

Infection prevention regimen haematology ward

At the haematology ward of our hospital, selective decontamination of the digestive tract (SDD) is performed in patients with an (expected) reversible neutropenia or increased risk of infection. The implementation is as follows: surveillance cultures from faeces, throat and urine at admission day, then once a week only faeces and throat cultures during the duration of neutropenia. Penicillin (to prevent streptococcal sepsis) and ciprofloxacin or cotrimoxazol or colistine or tobramycin (orally) are used as prophylactic antibiotics depending on the resistance pattern of surveillance cultures. Amphoterin B, nystatin or fluconazole are given orally as antifungal therapy. The choice of empirical antibiotic therapy is piperacillin-tazobactam.

Screening for *E. faecium* in this period was done on BME(G) agar plates. This contained Meropenem 64mg/L, Oxacillin 10mg/L, Amphotericin-B 20mg/L and esculin. Hereby we screened for ampicillin resistant *E. faecium* (HA *E. faecium*). From January 2007 these agar plates also contained gentamicin 128mg/L since there was an increase of high level gentamicin resistant *E. faecium* in our hospital from that time period.

Identification and susceptibility testing

Blood cultures were performed using the BACTEC system (Becton Dickinson™). Further determination and susceptibility testing were performed for gram positive streptococci that were catalase negative and PYR positive. As for *E. faecium* surveillance cultures, only colonies that grew on the BMEG plates with black borders were further determined. Species were identified using the VITEK®2 System (BioMérieux™) or API20 Strep System (BioMérieux™). Subsequently antimicrobial susceptibility testing was performed using the VITEK®2 System or disk diffusion tests respectively.

Statistical analyses

Statistical analyses were performed using SPSS for Windows, rel 18.0. Univariate analyses were performed using the Fisher's exact or Chi-square methods for categorical variables.

The Student's t-test or Mann-Whitney *U*-test was used for the continuous variables. Results with a *p*-value of ≤0.05 were considered to be statistically significant. All *p*-values are two-tailed. Significant variables were used in the multivariate logistic regression.

Deriving prediction model from a nested case-control design

To overcome the overestimation of risks because of overrepresentation of cases, we choose to perform a nested case-control design where the cases represent 5% and controls 95% of the whole population (Figure 1). Therefore the following factor to the intercept of the logistic regression model is added: c=ln (q_0/ (1-q_0)), whereas q_0 is the true prevalence of the diseases in the population. With this correction the risk of an individual to get the disease can be estimated by the formula $e^{\beta 0+c+\ \beta 1X1+\dots\beta kXk}$ / 1+e $^{\beta 0+c+\ \beta 1X1+\dots\beta kXk}$. In this formula, β_0 is the intercept from the linear regression equation, β_1/β_k is the regression coefficient derived from the multivariate logistic regression and X_1/X_k is the value of the predictor. In this study, q0 is the prevalence of patients with an *E. faecium* blood culture. Since we were only interested in those patients of whom a blood culture is drawn, c=ln (0.05/ (1-0.05)) =-2.94. Controls should be a random selection representative of the population [14] which is the case since we randomly selected the 66 control patients.

RESULTS

Patients

A total of 99 patients were evaluated: 33 cases (*E. faecium*) and 66 controls. Characteristics of the 66 controls showed the following blood culture results: *E. coli* (n=4), *Streptococcus viridans* (n=2), CoNS (n=4), *Corynebacterium* spp. (n=1) and no growths (n=55). Comparisons of the demographic and clinical data are presented in Table 1. There were no significant differences between type or status of disease. Patients with *E. faecium* bacteremia were associated with higher age and longer hospitalization days prior to blood culture as well as one year before admission. They were also associated with severe and longer duration of neutropenia, longer duration of fever and higher CRP levels at time of blood culture withdrawal. Penicillin and quinolones as a part of the SDD regimen and piperacillin-tazobactam as empirical broad-spectrum antibiotics were the most frequently used antibiotics; however this did not differ between the two groups. Only "other" antibiotics were more frequently given in the *E. faecium* group. This was mainly colistine, a polymixin antibiotic, though colistine use alone was not significant.

Table 1: Comparison of demographic and clinical characteristics of cases (*E. faecium*) and controls

Demographics	Cases (*n*=33)	Controls (*n*=66)	*p*-value
Male gender	18 (68.2%)	45 (54.5%)	0.184
Age, mean ± SD, years	58.0 ± 11.3	52.2 ± 9.1	0.008
Type of malignancy: [a]			0.378
– Leukaemia (AML, MDS, ALL) for chemotherapy	19 (57.6%)	28 (42.6%)	
– Leukemia for allogeneic stem cell transplantation	2 (6.1%)	2 (3.0%)	
– Lymphoma's, Kahler, CLL and others undergoing autologous stem cell transplantation	6 (18.2%)	17 (25.8%)	
– Lympfhoma's, Kahler, CLL not undergoing autologous stem cell transplantation	6 (18.2%)	19 (28.8%)	
Status of disease:			
– Remission	9 (27.3%)	11 (16.7%)	0.215
– Not in remission [b]	24 (72.7%)	55 (83.3%)	0.215
– Relapse	7 (21.2%)	14 (21.2%)	1.000
Reason for admission:			0.476
– Infection	4 (12.1%)	13 (19.7%)	
– Chemotherapy	21(63.6%)	34 (51.5%)	
– Stem cell transplantation [c]	8 (24.2%)	19 (28.8%)	
Length of hospital stay:			
– Length in days prior to positive blood culture, median (range)	21 (2-52)	13.5 (1-84)	0.007
– Length in days 1 year before admission, median (range)	43 (6-131)	24 (1-133)	0.018
Signs of organ failure: [d]			
– Renal (creatinine > 176µmol/L)	2 (6.1%)	3 (4.5%)	0.746
– Hepatic (bilirubin >34mmol/L)	2 (6.1%)	0 (0.0%)	0.109
– Lung (bilateral lung infiltrates)	4 (12.1%)	10 (15.2%)	0.769
Days of fever, median (range) [d]	2 (0-7)	0 (0-6)	0.001
Neutropenia:			
– Neutropenia <0.1x10^9/L [e]	20 (60.6%)	19 (28.8%)	0.002
– Neutropenia <0.5x10^9/L [e]	28 (84.8%)	28 (42.4%)	<0.001
– Neutropenia <2.0x10^9/L [e]	29 (87.9%)	39 (59.1%)	0.004
– Duration of neutropenia <0.5x10^9/L prior to blood culture, median (range)	8.0 (0-27)	0.0 (0-26)	<0.001
CRP (C-reactive protein in mg/L):			
– Levels 7 days prior to blood culture, median (range)	26 (3-263)	47 (5-347)	0.07
– Levels at time of blood culture, median (range)	188 (7-288)	108 (3-426)	0.006
– At time of blood culture CRP >125 mg/L	23 (69.7%)	24 (36.4%)	0.002

Demographics	Cases (*n*=33)	Controls (*n*=66)	*p*-value
Antibiotic therapy at time of blood culture and/or 30 days before:			
– Penicillins	24 (72.7%)	40 (60.6%)	0.234
– Cotrimoxazole	12 (36.4%)	18 (27.3%)	0.353
– Quinolones	25 (75.8%)	51 (77.3%)	0.866
– Cephalosporins	6 (18.2%)	4 (6.1%)	0.079
– Carbapenems	6 (18.2%)	5 (7.6%)	0.113
– Others [f]	19 (57.6%)	16 (24.2%)	0.001

[a] AML= acute myeloid leukaemia, MDS= myelodysplastic syndrome, ALL= acute lymphoblastic leukaemia, CLL= chronic lymphoblastic leukaemia [b] Including patients partially in remission [c] Allogeneic as well as autologous stem cell transplantation [d] At the day of blood culture till 7 days prior to blood culture [e] At the day of blood culture withdrawal [f] colistin, tetracyclin, macrolides, aminoglycosides, metronidazole

Microbes

From the 33 cases, fourteen patients (42.4%) had a single blood culture, nineteen (57.6%) had more than one blood culture and 11 (33.3%) had more than two blood cultures. All *E. faecium* blood isolates were resistant to amoxicillin. No VRE strains were identified in this study. High-level gentamicin resistance (HLGR) was found in 19 (57.6%) of the 33 *E. faecium* blood isolates. Three of the 19 patients with HLGR *E. faecium* also had low level gentamicin resistant *E. faecium* in their blood cultures (multiple blood cultures).

Comparisons of the microbial data are presented in Table 2. Polymicrobial infections were found in 9.1% of the cases compared to 1 (1.5%) in the control group (p=0.107). Pathogens isolated were *Clostridium perfringens*, *Pseudomonas aeruginosa*, and Streptococcus species. Three case patients (9.1%) had a positive Galactomannan compared to 2 (3.0%) in the control group (p=0.330).

An abdominal focus was found to be associated with *E. faecium* bacteremia (p=0.003) of which diarrhea appeared to be most distinct variable. Only one patient with an *E. faecium* BSI had a positive *Clostridium difficile* toxin test (no *C. difficile* in stool culture) at time of diarrhea. This was two days prior to the positive blood culture, together with a positive *E. faecium* faeces culture, though this patient was already colonized with *E. faecium* for several weeks.

Table 2: Comparison of the microbiological characteristics of cases (*E. faecium*) and controls

	Cases (*n*=33)	Controls (*n*=66)	*p*-value
Colonization with *E. faecium* [a]			
– 7 days prior to blood culture	13 (39.4%)	10 (15.2%)	0.007
– 30 days prior to blood culture	19 (57.6%)	14 (21.2%)	<0.001
– 90 days prior to blood culture	21 (63.6%)	16 (24.2%)	<0.001
– Number of faeces cultures with *E. faecium* 30 days prior to blood culture, median (range)	1 (0-8)	0 (0-6)	<0.001
Type of blood culture			
– Polymicrobial [b]	3 (9.1%)	1 (1.5%)	0.107
– Galactomannan	3 (9.1%)	2 (3.0%)	0.330
Clinical source of infection			
– CVC presence	26 (78.8%)	43 (65.2%)	0.164
– Abdominal focus: abdominal pain and/or diarrhea	25 (75.8%)	29 (43.9%)	0.003
– Abdominal pain	9 (27.3%)	11 (16.7%)	0.215
– Diarrhea	23 (69.7%)	26 (39.4%)	0.004
– Mucositis	13 (39.4%)	18 (27.3%)	0.220
– Lungs			
– Coughing and/or sputum	8 (24.2%)	15 (22.7%)	0.866
– Radiological proof of pneumonia or lung infiltrates	4 (12.1%)	14 (21.2%)	0.269
– Ear Nose Throat	1 (3.0%)	2 (3.0%)	1.000
– Skin	7 (21.2%)	19 (28.8%)	0.419
– Urinary infection	1 (3.0%)	9 (13.6%)	0.158

[a] In faeces culture, part of the SDD regimen [b] Within ± 7 days, less pathogenic micro-organisms (coagulase-negative staphylococci, corynebacteria, micrococcus spp. and bacillus spp.) are excluded.

Patients with *E. faecium* BSI were more often detected to be colonized with *E. faecium* prior to blood culture (p=<0.001). A total of twenty-one patients (63.6%) were colonized with *E. faecium* prior to the positive blood culture with a median of 1 (range 0-8), compared to 24.2% in the control group with a median of 0 (range 0-6). Twelve patients (36.4%) were not found to be colonized with the surveillance cultures. However, nine of these twelve patients had a blood culture with low level gentamicin resistant *E. faecium*. Seven of these twelve patients (58.3%), had a positive faeces culture with *E. faecium* after all within 30 days after positive blood culture; five with high level gentamicin resistant *E. faecium*, two with low level gentamicin resistant *E. faecium*. The majority of the patients (69.7%) were still colonized up to 30 days after the first positive blood culture. This includes both patients that were already colonized and patients who had a positive culture with *E. faecium* within 30 days after their positive blood culture.

Outcomes and treatment

Both groups had an equal antibiotic treatment for piperacillin-tazobactam as well as for glycopeptide treatment at time of blood culture withdrawal. (Table 3) Patients with an *E. faecium* BSI were more often admitted to the ICU after the positive blood culture. Reasons for ICU admissions were predominantly sepsis, mostly with an abdominal focus (abdominal sepsis). The 7-day mortality as well as the 30-day mortality were significantly higher in patients with *E. faecium* BSI compared to the control group (30.3% vs 4.5%; *p*=0.001 and 39.4% vs 10.6%; *p*=0.001 respectively). All 10 patients with *E. faecium* BSI that died within 7 days after their last positive culture were diagnosed with sepsis or severe infection, six of them (60%) had an clear abdominal focus (abdominal sepsis). Another three patients died after 30 days, one diagnosed with a septic shock, the other two patients had multiple diagnoses.

Table 3: Comparison of outcome and antibiotic treatment of cases (*E. faecium*) and controls

	Cases (*n*=33)	Controls (*n*=66)	*p*-value
Piperacillin-tazobactam treatment at time blood culture is drawn and/or 30 days before	22 (66.7%)	42 (63.6%)	0.766
Vancomycin/teicoplanin treatment at time of blood culture withdrawal	4 (12.1%)	8 (12.1%)	1.000
ICU admission till 7 days after positive bloodculture	5 (15.2%)	1 (1.5%)	0.015
Mortality*			
– At 7 days	10 (30.3%)	3 (4.5%)	0.001
– At 30 days	13 (39.4%)	7 (10.6%)	0.001

*After last positive blood culture with *E. faecium*

More detailed data considering antibiotic treatment in patients with an *E. faecium* BSI including mortality rates are presented in Table 4. Only 4 patients (12.1%) received glycopeptide treatment at time of blood culture withdrawal. Three of them had an empirical treatment and one received treatment because of an earlier proven CoNS infection. After 24 hours a total of 19 patients (57.6%) received glycopeptide treatment. Of these 19 cases, four were empirically treated upfront because of septic profile, two cases because of a CoNS infection and 13 cases recommended by the medical microbiologist because of suspected or proven *E. faecium* blood culture. Still, fourteen patients (42.4%) had no adequate treatment for their infection after 24 hours.

Table 4: Antibiotic treatment with vancomycin or teicoplanin in patients with *E. faecium* BSI, including mortality rates (*n*=33)

	Vancomycine/teicoplanin treatment cases (*n*=33)					
	Yes				**No**	
	Empirical	Mortality	Therapeutic	Mortality		Mortality
At time of blood culture withdrawal	3 (9.1%)	2/3 (66.7%)	1* (3%)	0 (0%)	29 (87.9%)	11/29 (37.9%)
After 24 hrs	4 (12.1%)	3/4 (75%)	13+2* (45.5%)	5/15 (33.3%)	14 (42.4%)	5/14 (35.7%)

*Because of coagulase negative staphylococci

Additional statistical analyses were performed on patients with an *E. faecium* BSI (cases) to determine additional risk factors for mortality. Only the numbers of blood cultures were found to be statistically significant for mortality at 7 days, with significant trend effect in case of more positive blood cultures. (Additional supplement 1) None of the other demographic, clinical or microbiologic factors listed in Table 1 and 2 (e.g. neutropenia, mucositis, glycopeptide treatment) were found to be additional risk factors.

Multivariable regression analysis and prediction modeling

Variables included in the multivariate regression analyses are shown in Table 5. Independent risk factors for an *E. faecium* BSI are colonization with *E. faecium* 30 days prior to blood culture (OR 5.71; CI 1.7-18.7), combination of neutropenia and abdominal focus (4.37; 1.4-13.4), age > 58 years (4.01; 1.3-12.5), hospital stay prior to blood culture > 14 days (3.55; 0.98-12.9) and CRP (C-reactive protein) level >125mg/L (4.37; 1.1-10.2).

Subsequently these independent risk factors were used in order to develop the prediction model. A subset of this prediction model is shown in Table 6. Hereby the formula $e^{\beta 0+c+ \beta 1X1+\ldots \beta kXk} / 1+e^{\beta 0+c+ \beta 1X1+\ldots\beta kXk}$ was used, whereas β was deduced from the multivariate regression analysis as shown in Table 5. Since five variables were tested and used in this model, a total of 32 outcomes are possible. If a patient has all the five variables at the moment of blood culture withdrawal, the risk of an *E. faecium* BSI is 47.5%. If a patient has none of the variables the risk is close to zero. In clinical decision making the clinician can fill in the variables; 0 for a negative and 1 for a positive score and thereby deduce the risk of *E. faecium* BSI. (All 32 variables and probabilities are available in an additional supplement Table S1)

Table 5: Multivariate logistic regression analyses: risk factors associated with an *E. faecium* BSI (*n*=33)

Variables tested	B	*p*	OR	[95% CI]
A. Colonization with *E. faecium* 30 days prior to blood culture	1.742	0.004	5.71	[1.7-18.7]
B. Neutropenia and abdominal focus*	1.474	0.010	4.37	[1.4-13.4]
C. Age > 58 years	1.390	0.017	4.01	[1.3-12.5]
D. Days of admission prior to blood culture > 14 days	1.267	0.054	3.56	[0.98-12.9]
E. CRP >125mg/L	1.216	0.032	4.37	[1.1-10.2]

*Abdominal pain and/or diarrhea. B=regression coefficient. *P*=*p*-value. OR=Odds ratio. 95% CI=95% confidence interval

Table 6: Prediction model to determine the risk of *E. faecium* BSI (subset)

Variables tested	B	*p*	OR	[95% CI]
A. Colonization with *E. faecium* 30 days prior to blood culture	1.742	0.004	5.71	[1.7-18.7]
B. Neutropenia and abdominal focus*	1.474	0.010	4.37	[1.4-13.4]
C. Age > 58 years	1.390	0.017	4.01	[1.3-12.5]
D. Days of admission prior to blood culture > 14 days	1.267	0.054	3.56	[0.98-12.9]
E. CRP >125mg/L	1.216	0.032	4.37	[1.1-10.2]

For this prediction model the formula $e^{\beta 0+c+\beta 1X1+\ldots\beta kXk} / 1+e^{\beta 0+c+\beta 1X1+\ldots\beta kXk}$ was used, whereas β was deduced from the multivariate regression analysis as shown in table 5. 0 = variable absent, 1 = variable present A=Colonization with *E. faecium* 30 days prior to blood culture B=Neutropenia and abdominal focus (diarrhea or abdominal pain) C=Age over 58 years D=Days of admission prior to blood culture more than 14 days E=CRP >125mg/L

DISCUSSION

Nowadays *E. faecium* has become an emerging and challenging pathogen in hospitals and even more has replaced *E. faecalis* as the predominant enterococcus species [8]. The increase of *E. faecium* BSIs in our study are in line with the numbers of a recent EARSS (European Antimicrobial Resistance Surveillance System) study, in which *E. faecium* increased most significant in BSIs compared to other major pathogens [15].

All *E. faecium* strains from the blood cultures in our study belonged to the HA/CC-17 strains. They were all amoxicillin (ampicillin) resistant and insertion sequence 16 (*IS*16) positive, which is a marker for these strains [16]. HA/CC-17 strains seem to be successful in acquiring accessory virulence and antibiotic genes and therefore might set the stage for VRE [17]. In vancomycin resistant *E. faecium* infections, adequate treatment of serious infections becomes limited. Although some novel antimicrobials such as linezolid and

daptomycin have been developed, these also have their limitations; moreover resistance to these antimicrobials has already been described [18].

In line with previous studies prior colonization with HA *E. faecium* showed to be an independent risk factor for *E. faecium* BSI. [19, 20] This study showed that the majority of patients (63.6%) were first colonized prior to the development of *E. faecium* BSI; moreover it seemed to be the most important/significant independent risk factor for *E. faecium* BSI in our study. It is important to keep in mind that multiple swabs might be needed to detect the majority of carriers [21] and *E. faecium* can persist for a long period [22] which is also seen in our study. Environmental contamination and person-to-person spread are factors contributing to the acquisition of *E. faecium* [23, 24]. *Enterococcus* spp. are quite tenacious and may survive for more than 4 months under dry conditions [25]. Therefore standard hygiene (e.g. hand hygiene) and appropriate infection-control measures (e.g. risk surface disinfection) are essential.

Neutropenia and abdominal focus (diarrhea and/or abdominal pain) were also associated with *E. faecium* BSI. Because these variables seem to be related to each other, as they individually excluded each other in regression analysis, the two variables were combined. The extensive chemo- and transplantation therapy the patients receive is often associated with neutropenia and diarrhea [26]. In case of severe neutropenia or chemotherapy induced diarrhea which can be seen as injury of the mucosal barrier, *E. faecium* has the opportunity to enter the bloodstream.

Subsequently we expected mucositis, which relates more to the oral toxicity of chemotherapy, to be an associated variable. Kuehnert et al. showed that the risk of VRE BSI increased with increasingly severe mucositis [27]. In contrast, Worth et al didn't find mucositis to be associated with *E. faecium* infection; however it hadn't a well-validated mucositis severity index [28]. Perhaps a more validated mucositis stratification would have shown other results in our study.

CRP level and fever as infection parameters were both found to be significant. However, they individually excluded each other in the regression analysis. Therefore we chose to include CRP level in our model as it is a more objective parameter. Especially in these haematology patients, fever can be aspecifically related to for example drug fever or inflammation like mucositis.

Not many studies have identified age to be an independent risk factor. However the majority of the patients with *E. faecium* infections in the studies are at higher age (50-70 years) and these studies included a more specific control group [11, 29, 30] whereas we

choose a random selection representative for the total population of the haematology ward during the study period.

Since *E. faecium* is considered to be a nosocomial pathogen, a prolonged hospital length of stay as a predictor in *E. faecium* bacteremia is as we expected. For VRE as a multi-resistant pathogen it is clear it is associated with a longer hospital length of stay. Though also for vancomycin-susceptible (VSE), but ampicillin resistant *E. faecium* (ARE) as in our study, this association had been shown [31, 32].

Another risk factor often associated with *E. faecium* infection is previous antibiotic use [30]. Moreover, numbers of enterococci in SDD increases since they are not covered [33]. We haven't found a strong association between antibiotic use and an *E. faecium* BSI, since the majority of both patient groups received SDD.

Additional analysis between patients with and without an *E. faecium* BSI did not result in additional risk factors for mortality besides the total number of positive *E. faecium* blood cultures. However numbers were often too small to perform adequate statistical analyses between the two groups.

This study has some limitations. Firstly, the data was retrospectively gathered. Although both stored-hard copy and electronic records were reviewed, for certain clinical parameters precise monitoring was difficult. Secondly, this is a single-centre study whereas local epidemiological variables and infection prevention measures must be considered. Thirtly, from January 2007 surveillance cultures were screened for meropenem and high level gentamicin resistant *E. faecium*. The reason for this was an increase in *E. faecium* of which the major part was high level gentamicin resistant in our hospital from that time period. An unknown number of *E. faecium* of gentamicin susceptible surveillance cultures have been missed during this period. However, we still detected some gentamicin low level resistant *E. faecium* (5/200 patients ~5%) from that time period. From February 2011 we use 2mg/L gentamicin in our BMEG screening agars instead of 128mg/L. Hereby we see an increase of ~30% due to low level gentamicin *E. faecium* in the haematology ward for the period February 2011 – July 2013. However, there seems to be a shift again from 2012, whereas gentamicin high level *E. faecium,* accounts for up to 80% of the HA *E. faecium* both in screening cultures as well as in blood cultures for the period February 2012 – July 2013. This should be taken into account considering results of *E. faecium* colonization in our study. It is difficult to assess the implication of this limitation on the prediction model with respect to the odds ratio. Moreover, patients can have several *E. faecium* strains in their surveillance cultures as well as in blood cultures. Finally the majority of our control group had blood

cultures with 'no growths'. This might have several reasons, for example patients could have had fever due to the malignancy or drug fever or inflammation because of mucositis. It could also partially be explained by the fact patients received SDD. One can state that these patients had a lower degree of illness, compared to patients with an *E. faecium* blood culture. However, retrospective circumstances for both groups were equal. Both groups had the same grounds to obtain a blood culture; neutropenic fever or other clinical signs of infection. Also for the purpose of the study, a prediction model in order to decide whether or not add glycopeptide to the empirical antibiotic treatment at the moment a blood culture is drawn, we choose to select this group of patients as controls.

In conclusion this study demonstrated that colonization with HA *E. faecium* 30 days prior to blood culture, combination of neutropenia and abdominal focus, age > 58 years, hospital stay prior to blood culture > 14 days and CRP level >125mg/L are independent risk factors for *E. faecium* BSI. In agreement with previous studies, this study showed that *E. faecium* infections can cause severe infections and are associated with high mortality rates in patients with haematologic malignancies [10, 34]. Thereby risk stratification becomes necessary in those haematology patients at high risk. Using a prediction model for risk stratification as designed in this study, antibiotic stewardship in terms of prudent use of glycopeptides becomes possible. Together with infection control measures this might be helpful controlling further increase of VRE. The prediction model in this study is based on one specific haematology ward, though it would be worthwhile to verify this prediction model in a prospective multicenter study.

Authors' contribution

XZ has contributed to the conception and design of the study, gathered laboratory and clinical data, analyzed the data and drafted the original article. JA contributed to the conception and design of the study, gathered laboratory data and revised the article. LS contributed to the conception and design of the study and critically revised the article. AF critically revised the article.

Acknowledgements

This work was supported by the Interreg IVa-funded projects EurSafety Heath-net (III-1-02=73) and SafeGuard (III-2-03=025), part of a Dutch-German cross-border network supported by the European Commission, the German Federal States of Nordrhein-Westfalen and Niedersachsen, and the Dutch provinces of Overijssel, Gelderland, and Limburg.

We thank I.M. Nolte (University Medical Center Groningen, Department of Epidemiology) and T. Donker (University Medical Center Groningen, Department of Medical Microbiology) for the excellent help and discussion about the statistical analysis of the data. We thank G.A. Kampinga for the great help and knowledge on microbiological methods used at the laboratory.

Transparency declarations

None to declare

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Table S1: Association between numbers of *E. faecium* blood cultures and mortality in patients with an *E. faecium* BSI/cases (*n*=33)

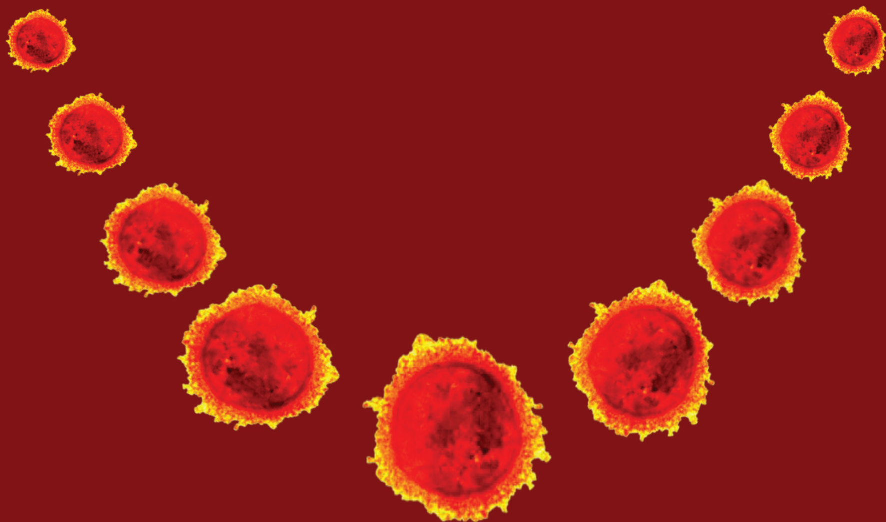
Mortality at 7 days	Yes (n=10)	No (n=23)	*p*-value
Numbers of *E. faecium* blood cultures, median (range)	3.5 (1-10)	1 (1-12)	0.05
More than one *E. faecium* blood culture	9 (90%)	10 (43.5%)	0.02
More than two *E. faecium* blood cultures	7 (70%)	4 (17.4%)	0.006

Mortality at 30 days	Yes (n=13)	No (n=20)	*p*-value
Numbers of *E. faecium* blood cultures, median (range)	3.0 (1-10)	1.5 (1-12)	0.127
More than two *E. faecium* blood cultures	7 (53.8%)	4 (20%)	0.065

Table S2: Complete prediction model to determine the risk of *E. faecium* BSI

A	B	C	D	E	Probability
1	1	1	1	1	47.5
1	1	0	1	1	18.4
1	0	1	1	1	17.2
0	1	1	1	1	13.7
1	1	1	0	1	20.3
1	1	1	1	0	21.2
1	0	0	1	1	4.9
0	1	0	1	1	3.8
1	1	0	0	1	6.0
0	0	1	1	1	3.5
1	1	0	1	0	6.3
1	0	1	0	1	5.5
0	1	1	0	1	4.3
1	0	1	1	0	5.8
0	1	1	1	0	4.5
1	1	1	0	0	7.0
0	0	0	1	1	0.9
1	0	0	0	1	1.4
0	1	0	0	1	1.1
1	0	0	1	0	1.5
0	1	0	1	0	1.2
0	0	1	0	1	1.0
1	1	0	0	0	1.8
0	0	1	1	0	1.1
1	0	1	0	0	1.7
0	1	1	0	0	1.3
0	0	0	0	1	0.25
0	0	0	1	0	0.27
0	1	0	0	0	0.33
1	0	0	0	0	0.43
0	0	1	0	0	0.30
0	0	0	0	0	0.08

For this prediction model the formula $e^{\beta 0+c+ \beta 1X1+\dots\beta kXk}$ / $1+e^{\beta 0+c+ \beta 1X1+\dots\beta kXk}$ was used, whereas β was deduced from the multivariate regression analysis as shown in table 5. 0 = variable absent, 1 = variable present. A= Colonization with *E. faecium* 30 days prior to blood culture B= Neutropenia and abdominal focus (diarrhea or abdominal pain) C= Age over 58 years D= Days of admission prior to blood culture more than 14 days E= CRP >125mg/L



Evaluation of the Xpert *vanA/vanB* assay using enriched inoculated broths for the direct detection of *vanB* VRE

5

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Keywords: *Enterococcus faecium*, VRE, *vanB*, GeneXpert, Real-time PCR, Infection control

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ABSTRACT:

Rapid and accurate detection of VRE (vancomycin resistant enterococci) is required for adequate antimicrobial treatment and infection prevention measures. Previous studies using PCR for the detection of VRE, including the Cepheid's Xpert *vanA*/*vanB* assay, reported accurate detection of *vanA* VRE, however many false positive results were found for *vanB* VRE. This is mainly due to non-enterococcal *vanB* genes which can be found in the gut flora. Our goal was to optimize the rapid and accurate detection of *vanB* VRE and to improve the positive predictive value (PPV) by limiting false-positive results. We evaluated the use of the Xpert *vanA*/*vanB* assay on rectal swabs and on enriched inoculated broths for the detection of *vanB* VRE. By adjusting the cut-off CT-value to ≤ 25 for positivity by PCR on enriched broths, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) resulted in 96.9%, 100%, 100% and 99.5% for *vanB* VRE, respectively. As shown in this study CT-values ≤ 25 acquired from enriched broths can be considered as true-positive. For broths with CT-values between 25-30, we recommend to confirm this by culture. CT-values of >30 appeared to be true-negative. In conclusion, this study shows that the Cepheid's Xpert *vanA*/*vanB* assay performed on enriched inoculated broths with an adjusted cut-off CT-value is an useful and rapid tool for the detection of *vanB* VRE.

INTRODUCTION

Vancomycin resistant enterococci (VRE) have emerged as an important nosocomial problem worldwide. The rise of VRE is mostly due to *Enterococcus faecium*, with *vanA* and *vanB* being the two clinically most important genotypes [1]. VanA-type resistance is induced by teicoplanin and vancomycin causing resistance to both antibiotics. In contrast, VanB-type resistance is only induced by vancomycin, resulting in variable levels of vancomycin resistance but still being susceptible to teicoplanin [2].

Rapid and accurate detection of VRE is required for adequate antimicrobial treatment and infection prevention measures. Culture based methods to detect VRE are often time-consuming and take several days to complete (2-5 days). These time-consuming methods have a high economic impact on the infection control measures that has to be taken by the hospital, especially during outbreaks [3]. Several studies evaluated PCR-based methods for rapid detection of VRE including the Cepheid's Xpert *vanA*/*vanB* assay [4-7]. This assay runs on the Cepheid GeneXpert™ system, a fully automated processor that combines DNA extraction, real-time PCR amplification and detection, providing results within an hour. PCR-based methods are highly sensitive and specific for the detection of *vanA* VRE [7]. However, for *vanB* VRE many false positive results are reported, mainly due to non-enterococcal *vanB* genes which can be found in the gut, especially in anaerobic bacteria like *Clostridium species* [8-11]. Therefore, positive *vanB* VRE results still need to be confirmed by culture. An additional problem is that VanB-type resistance is sometimes difficult to detect since the vancomycin minimum inhibitory concentration (MICs) can be below the antimicrobial susceptibility breakpoint of ≤4 mg/L defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [12-14].

In our hospital, VRE screening is performed in patients coming from foreign hospitals, on ICU wards and in case of an unexpected VRE observation, e.g. if VRE is found in clinical specimens from epidemiologically linked patients. Isolation precautions are applied to patients coming from foreign hospitals at admission until patient samples are negative. During a (suspected) VRE outbreak patients are cohorted and screened on regular bases. Prior to this study, VRE detection was performed on in enriched inoculated broths with a conventional gel-based PCR. However, many false-postive results were obtained with this technique.

In March and April 2013 our hospital faced an outbreak with *vanB* VRE. During this outbreak we used and evaluated the Xpert *vanA*/*vanB* assay on rectal swabs and on enriched inoculated broths. Our goal was to optimize the rapid and accurate detection of *vanB* VRE and to improve the positive predictive value (PPV) by limiting false-positive results.

METHODS:

The University Medical Center Groningen is a 1300-bed tertiary care center. During an outbreak of *vanB* VRE in March and April 2013, rectal Eswabs (Copan ESwab™) were collected from hospitalized patients at the relevant wards for VRE testing.

Lab-study design:

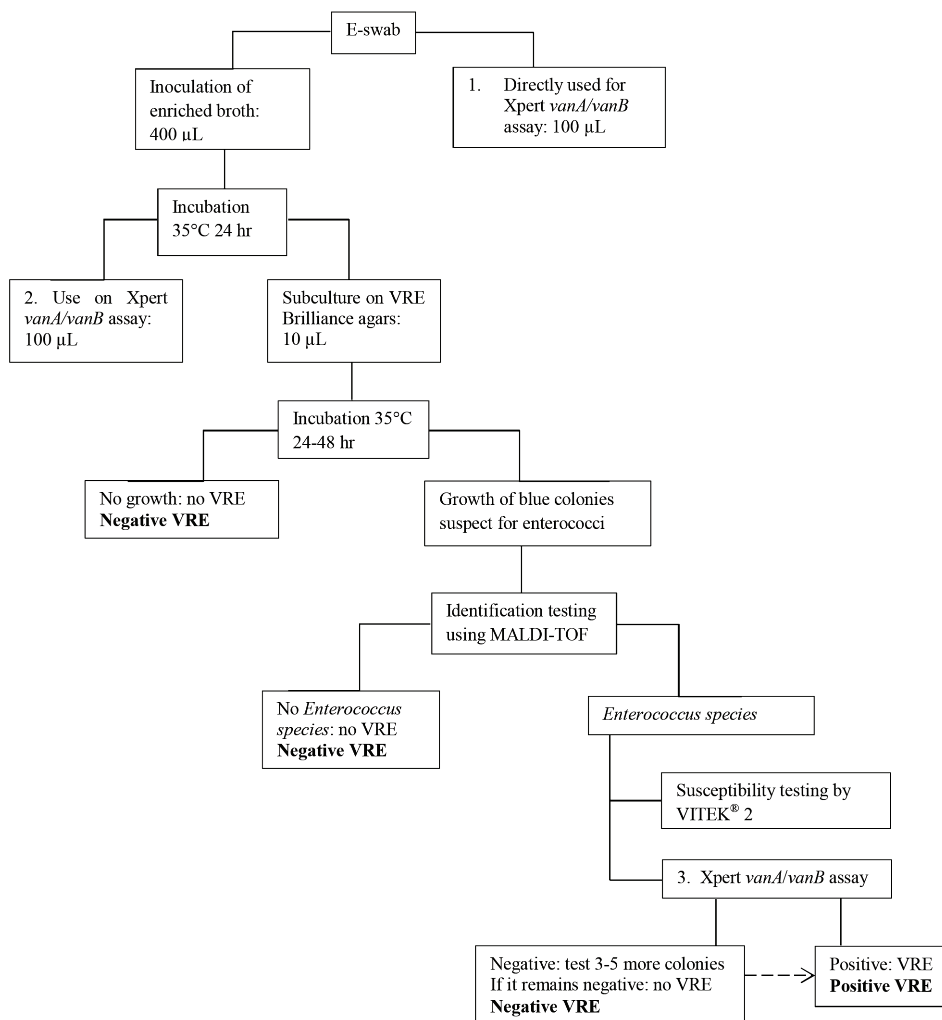
In total 235 Eswabs from 91 patients were used. The Xpert *vanA/vanB* assay was directly performed on 100 µL Eswab medium, and on enriched inoculated broths after 24 hours of incubation. For this latter, 9 mL of Brain Heart Infusion (BHI) broth containing amoxicillin 16mg/L, amphoterin-B 20 mg/L, aztreonam 20 mg/L and colistin 20 mg/L was inoculated with 400 µL Eswab medium and incubated at 35°C for 24 hours. Amoxicillin was used since VRE outbreaks are typically caused by amoxicillin resistant enterococci (ARE) that acquired resistance for vancomycin [15, 16]. Moreover, amoxicillin limits the growth of amoxicillin sensitive anaerobic bacteria like *Clostridium species* which are one of the most relevant species that may contain *vanB* genes [8, 17, 17]. Vancomycin was not added to the broth, as this would hamper the detection of *vanB* VRE expressing low vancomycin MICs [12, 14]. From the broths, 100 µL was used in the Xpert *vanA/vanB* assay and 10 µL of broth was subcultured on VRE Brilliance agars (Oxoid®). Agars were incubated at 35°C and examined after 24-48 hours. Blue colonies suspected for enterococci were identified by MALDI-TOF Mass Spectrometry (Bruker). Confirmed *Enterococcus species* were tested for antibiotic susceptibility using VITEK®2 (Biomerieux). The MIC clinical breakpoints defined by the (EUCAST) for *Enterococcus* spp. are as follows: for vancomycin, susceptible, ≤4 mg/L; resistant, >4 mg/L; for teicoplanin, susceptible, ≤2 mg/L; resistant, >2 mg/L [17]. Subsequently colonies were analyzed in the Xpert *vanA/vanB* assay. In case the Xpert vanA/van B assay was negative, 3-5 more colonies were tested (Figure 1).

The Cepheid GeneXpert™ system:

The Xpert *vanA/vanB* assay was performed on three different sources as depicted in Figure 1. For Eswab medium as well as for enriched inoculated broths, 100 µL was added to the elution buffer, vortexed for 10 seconds and transferred into the Xpert *vanA/vanB* cartridge. For isolates on VRE Brilliance agar, a suspension of 1-2 bacteria colonies was made using 1 mL milli-Q water (Sigma-Aldrich™), vortexed for 10 seconds and then diluted 1:500 in milli-Q water. Subsequently, 75 µL of the diluted sample was added to the elution buffer and transferred to the Xpert *vanA/vanB* cartridge. The amounts used for the Xpert *vanA/vanB* assay were

advised by the manufacturers' and/or validated in our laboratory. Further procedures were performed according to the manufactures' guidelines (Xpert *vanA/vanB* 301-0188). According to these guidelines CT-values of ≤36 are considered to be positive, whereas CT-values of >36 are considered to be negative. A CT-value of 0 for *vanA* and *vanB* indicates no amplification and is considered to be negative if the internal control present in the assay is valid.

Figure 1: Workflow of the study, including definitions of negative and positive VRE indicated in bold (gold standard). Numbers 1, 2 and 3 reflect the three different sources used in the Cepheid's Xpert *vanA/vanB* assay.



Resolution of discordant results:

As mentioned before, VRE detection is difficult and different detection methods are used. The use of a direct rectal swab culture method for the detection of VRE colonization shows a high rate of false-negative results [18]. Culture of rectal samples after broth enrichment followed by species identification and susceptibility testing is most sensitive for detecting VRE [19]. Therefore, we defined a sample as true-positive for VRE when the Xpert assay performed on the isolate from the VRE Brilliance agar, after broth enrichment, was positive (our gold standard). A sample was considered as true-negative for VRE in case of the following results 1) observation of no growth on VRE Brilliance agar after broth enrichment, 2) growth of species other than *Enterococcus species* on the VRE Brilliance agar 3) growth of *Enterococcus species* on VRE Brilliance agar but negative in the Xpert assay performed on the isolate (Figure 1).

The new algorithm

Based on the CT-values acquired from Eswab medium compared to those acquired from the enriched inoculated broths, we defined a new cut-off value for positivity by PCR on enriched broths. Broths with CT-values ≤ 25 were considered to be true-positive for *vanB* VRE, whereas broths with CT-values between 25-30 require confirmation by culture. Samples with CT-values of 0 (no amplification) or >30 were considered to be negative. To test our new algorithm, we prospectively evaluated 112 enriched inoculated broths from routine screenings.

Statistical methods:

Sensitivity, specificity, PPV and negative predictive values (NPV) were calculated for the results of the Xpert assay performed directly on Eswab medium as well as on enriched inoculated broths. The 95% confidence intervals were calculated using the Wilson 95% confidence interval including continuity correction [20].

RESULTS:

Out of 235 Eswabs, 157 were negative in the Xpert *vanA/vanB* assay and confirmed to be true-negative for VRE according to our definitions (Table 1). In these 235 Eswabs no *vanA* VRE was found. A total of 78 Eswabs were *vanB* VRE positive according to the assay, of which 32 were confirmed to be true-positive for *vanB* VRE according to our definition. Moreover, all 32 isolates were identified as *E. faecium* and had a typical VanB phenotype by VITEK®2 susceptibility testing. The MIC values is these 32 isolates ranged from 8mg/L to >32mg/L

for vancomycin, for teicoplanin all MIC values were <0.5 mg/L. The other 46 Eswabs were positive according to the assay, but no VRE could be confirmed using our gold standard and these were considered to be false-positive. Therefore, the Xpert assay on Eswabs resulted in a sensitivity, specificity, PPV and NPV of 100%, 77.3%, 41% and 100%, respectively (Table 2).

Table 1: Xpert *vanA/vanB* assay results using Eswabs and inoculated enriched broths with CT cut-off values for PCR positivity of ≤ 36 and ≤25 respectively, in relation to true VRE positivity and negativity.

	Eswabs (CT cut-off value ≤ 36)			**Inoculated enriched broths (CT-cut-off value ≤25)**		
	VRE positive*	**VRE negative***		**VRE positive***	**VRE negative***	
Xpert assay positive	32	46	78	31	0	31
Xpert assay negative	0	157	157	1	203	204
Total	32	203	235	32	203	235

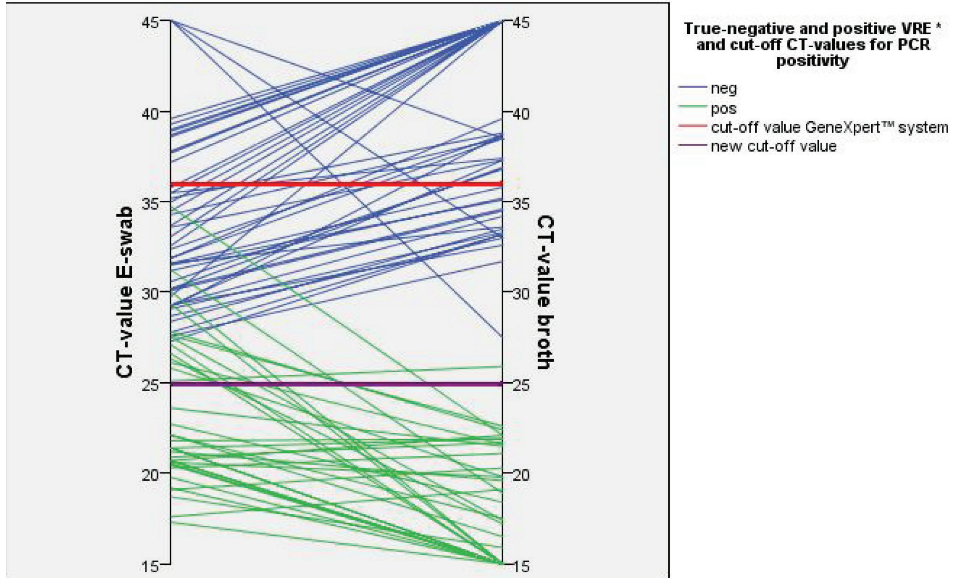
* See Material & Methods and Figure 1 for definitions.

Table 2: Sensitivity, specificity, PPVs and NPVs (95% confidence interval) of Eswab and inoculated enriched broth used in the Cepheid Xpert *vanA/vanB* PCR.

Method	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
Eswab (CT cut-off value ≤ 36)	100 (86.7-100)	77.3 (70.8-82.8)	41 (30.2-52.8)	100 (97.2-100)
Inoculated enriched broth (CT-cut-off value ≤25)	96.9 (82-99.8)	100 (97.7-100)	100 (86.3-100)	99.5 (96.9-100)

Using the Xpert assay on enriched broths resulted in a decrease of CT-values for the majority (80.6%) of true-positive cases compared to their CT-values obtained directly from Eswabs. For true-negative cases the opposite was observed for 94.7% of the samples (Figure 2). Because of the observed decline in CT-values of the broths we adjusted the cut-off value for PCR positivity of the Xpert assay on broth to ≤25. Sensitivity, specificity, PPV and NPV were recalculated and were 96.9%, 100%, 100% and 99.5%, respectively (Table 1 and 2). By using a cut-off value of ≤25 no false positive results were found, however, one true-positive VRE was missed (CT-value 25.9). Therefore, we defined a "gray-zone" for samples with a CT-value between 25-30 that require confirmation by culture. In this case, patients were not cohorted with VRE-positive persons until these samples were confirmed by culture. CT-values of >30 were considered to be true-negative. Importantly, our algorithm was prospectively tested using 112 enriched inoculated broths. We found 80 true-negative samples, 31 true-positive samples and one sample with a CT-value of 28.4 which required confirmation. The new algorithm resulted in a sensitivity, specificity, PPV and NPV of 100% for all.

Figure 2: Dynamics between CT values acquired by performing the Xpert vanA/vanB assay directly on ESwabs (left) and on enriched inoculated broths (right). One line represents one sample. Blue lines represent samples with confirmed true-negative VRE cultures. Green lines represent samples with confirmed true-positive VRE cultures. The red line indicates the cutoff CT value for PCR positivity used by the Cepheid GeneXpert system (≤36). The purple line indicates the new cutoff CT value for PCR positivity (≤25). In this figure CT values of 45 indicate that no amplification was detected (negative test).



* See Material & Methods and Figure 1 for definitions.

DISCUSSION:

In this study we evaluated the use of the Xpert *vanA/vanB* assay on rectal swab specimens and on enriched inoculated broths for the detection of *vanB* VRE. By using enriched broths combined with a new cut-off CT-value of ≤ 25 for PCR positivity, the PPV for VRE detection increased from 41% to 100%. As shown in this study CT-values ≤ 25 acquired from enriched broth can be considered as true-positive. For broths with CT-values between 25-30, we recommend to confirm this by culture. CT-values of >30 appeared to be true-negative.

VRE detection remains difficult and open for discussion regarding the best method to be used for the most reliable results. We are aware of the fact that use of feces is superior to a rectal swab as used in this study. However, use of feces is less practical for the clinicians during an outbreak screening. Some studies consider PCR-positive specimens to be true-positive even when results could not be confirmed by culture [21]. Nevertheless, we choose culture based methods after broth enrichments for our negative and positive VRE definitions and consider these methods to be the most valid in this study design.

The use of enriched broths containing amoxicillin still enables ARE to grow, whereas the growth of amoxicillin sensitive bacteria including anaerobes, like *Clostridium species,* are inhibited. These are the most important gut microorganisms that would otherwise interfere with the assay as they may contain non-enterococcal *vanB* genes [8-10]. A limitation of using amoxicillin is that the growth of *E. faecalis* is also inhibited. Therefore, it would be worthwhile to test the use of another agent such as metronidazole instead of amoxicillin in enriched broths. However, as noted earlier, the majority of VRE outbreaks are typically caused by ARE that acquired resistance for vancomycin [15, 16].

All *vanB* VRE isolates found in this study had a typical VanB phenotype as determined by VITEK®2. Remarkable, in this study no *vanB* VRE expressing low vancomycin MIC values were detected, although these strains have been found in our hospital in the past years. Since no vancomycin was added to the broth, we are convinced that detection of these *vanB* VRE strains were adequately performed. In addition, we observed that these strains grow on VRE Brilliance agars as was also shown by others [12].

As a consequence of using enriched broths instead of direct rectal specimens, results will become available 24 hours later. On the other hand, by using this method the PPV increases from 41% to 100% which is essential for right decision making with respect to infection prevention. To control an outbreak it is crucial to cohort true-negative patients apart from true-positives. An ongoing outbreak might require closure of the ward which has a high financial impact and subsequently an enormous impact on patient care. Samples with CT-values between 25-30 will take another 24-48 hours, though only the minority of samples in our study were amongst these values (0.6% of all samples). Therefore, the use of the Cepheid's Xpert *vanA*/*vanB* assay on inoculated enriched broths with an adjusted CT-value for PCR positivity can be considered as an useful and rapid tool for the detection of *vanB* VRE.

Acknowledgements

We would like to thank Dr. B.T.F. van der Gun for critical reading of the manuscript and helpful comments.

This study was supported by the Interreg IVa-funded projects EurSafety Heatlh-net (III-1-02=73) and SafeGuard (III-2-03=025), part of a Dutch-German cross-border network supported by the European Commission, the German Federal States of Nordrhein-Westfalen and Niedersachsen, and the Dutch provinces of Overijssel, Gelderland, and Limburg.

Transparency declarations

None to declare

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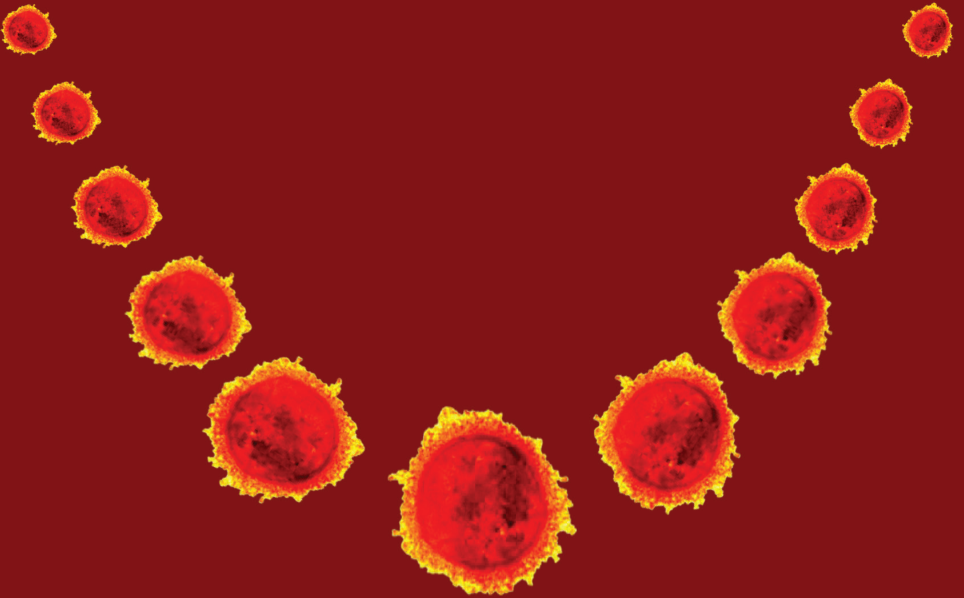

Diagnostic evasion of highly-resistant microorganisms: a critical factor in nosocomial outbreaks

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Running title: Diagnostic evasion of highly-resistant microorganisms in nosocomial outbreaks

Keywords: Diagnostic evasion, outbreaks, HRMO, signaling networks, antibiotic resistance

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ABSTRACT

Highly resistant microorganisms (HRMOs) may evade screening strategies used in routine diagnostics. Bacteria that have evolved to evade diagnostic tests may have a selective advantage in the nosocomial environment. Evasion of resistance detection can result from the following mechanisms: low-level expression of resistance genes not resulting in detectable resistance, slow growing variants, mimicry of wild-type-resistance and resistance mechanisms that are only detected if induced by antibiotic pressure.

We reviewed reports on hospital outbreaks in the Netherlands over the past 5 years. Remarkably, many outbreaks including major nation-wide outbreaks were caused by microorganisms able to evade resistance detection by diagnostic screening tests. We describe various examples of diagnostic evasion by several HRMOs and discuss this in a broad and international perspective.

The epidemiology of hospital-associated bacteria may strongly be affected by diagnostic screening strategies. This may result in an increasing reservoir of resistance genes in hospital populations that is unnoticed. The resistance elements may horizontally transfer to hosts with systems for high-level expression, resulting in a clinically significant resistance problem.

We advise to communicate the identification of highly resistant microorganisms that evade diagnostics within national and regional networks. Such signaling networks may prevent for inter-hospital outbreaks, and allow for collaborative development of adapted diagnostic tests.

INTRODUCTION

Diagnostic screening provides hospitals a level of immunity to antibiotic resistance. When highly resistant microorganisms (HRMOs) are detected, transmission can be limited by treating the patient with isolation precautions. In addition, the carriage of HRMOs can be suppressed by antibiotic treatment or, in case of methicillin resistant *Staphylococcus aureus* (MRSA), even be eradicated. If the introduction of HRMOs in hospitals remains undetected, these bacteria can disseminate from patient-to-patient, and the mobile genetic elements carrying resistance genes can horizontally transfer from species-to-species. Thus, the epidemiology of nosocomial resistance may strongly be affected by our diagnostic screening strategies. Moreover, we postulate that evasion of diagnostic resistance screening could be considered as a critical factor for infection of hospitals with antibiotic resistance elements, similar to the concept that immune evasion is a critical factor of pathogens to infect the human host.

The Netherlands is a high-resource country. Surveillance on HRMOs is extensive in Dutch hospitals. For this, the Dutch situation is very proficient to observe effects of diagnostic screening on the characteristics of HRMOs that cause nosocomial outbreaks. The Dutch Society for Medical Microbiology (NVMM) provides guidelines for the detection of HRMOs [1]. For the detection of HRMOs such as carbapenemase-producing Enterobacteriaceae (CPE), vancomycin-resistant enterococci (VRE), extended spectrum beta-lactamase (ESBL) - producing bacteria and MRSA, selective broth and/or selective media are used. Nosocomial outbreaks with HRMO are reported to 'Hospital Acquired Infection and Antimicrobial Resistance Monitoring Group" and the reports are communicated to clinical microbiologists. We searched these reports for outbreaks with micro-organisms harboring resistance mechanisms that were able to evade detection by routine diagnostics. In addition, we evaluated data from our hospital, and searched literature for outbreaks to assess the importance of diagnostic evasion. We here present the most explicit examples of CPE, VRE, ESBL-producing bacteria and MRSA outbreaks caused by isolates harboring diagnostic-evasive resistance mechanisms.

Diagnostic evasion by CPEs

In the Netherlands, the national laboratory guideline recommends the following screening strategy for the detection of highly resistant microorganisms: a screening step, a genotypic confirmation step and an optional phenotypic confirmation step [1, 2]. According to this guideline, *Enterobacteriaceae* with an MIC for meropenem ≥0.50 mg/L, or imipenem ≥2.0 mg/L should be evaluated by molecular tests for carbapenemase gene detection. Optional

phenotypic tests, which include the modified Hodge test, and tests based on inhibition of metallo-betalactamases by EDTA, and Class A carbapenemases by phenyl-boronic acid, may be used if genotypic confirmatory tests are not immediately available. Newer tests for non-genotypic detection of CPE include the carba-NP test, carbapenem-inactivation method (CIM-test), and immunochromotographic tests [3-6]. Genotypic confirmation comprises PCR and sequence based methods. Next-gen-sequencing facilities are increasingly accessible for routine diagnostic laboratories. This allows whole-genome sequence-based carbapenemase gene detection. In addition, specific primer/probe combinations for unique markers of an outbreak strains may be designed for high-throughput diagnostics to control outbreaks [7].

Despite this huge arsenal of CPE-detection methods, CPEs are still able to evade our diagnostic screening strategies. In the Netherlands, an inter-hospital outbreak with OXA-48-producing *Enterobacteriaceae* from 2009-2011 has been reported [8]. The outbreak had been uncontrolled for 2 years. The plasmids carrying $bla_{OXA\text{-}48}$ had disseminated to 15 (sub)-species. Predominantly OXA-48-producing *E. coli* and *K. pneumoniae* isolates were detected. Heterogeneity in resistance to carbapenems within, and across the OXA-48-producing species was observed. All OXA-48-producing *E. coli* isolates had meropenem MICs of <1 mg/L, a concentration that is commonly used in screening plates, whereas the meropenem susceptibility breakpoint for meropenem is 2mg/L according to EUCAST [9]. In addition, if the OXA-48 was not co-expressed with an ESBL gene, no hydrolysis of 3th generation cephalosporins was detected in the majority of isolates. These diagnostic stealth-features have undoubtedly contributed to the magnitude of this outbreak.

The emergence and spread of OXA-48 producing CPEs have been reported in several countries in Europe [10]. The outbreaks concerned predominantly *K. pneumoniae* clones. A successful *K. pneumoniae* clone carrying OXA-48 is ST 11, reported in many countries [11], amongst others in Greece [12], Spain [13] and Belgium [14]. Other clones associated with OXA-48 are ST14, ST15, ST101, SST 147 and ST405 [11, 13, 15]. In a Belgian multi-center study, less than 50% of CPEs were carbapenem non-susceptible [14].

Given the fact that OXA-48 is difficulty to detect, there is a need to adapt surveillance strategies to detect CPEs. The EUCAST-guideline advises to screen for CPE if isolates have a MIC to meropenem >0.12 mg/L [9]. Unfortunately, widely used automated susceptibility testing (AST) systems do not detect MICs below 0.5 mg/L. The meropenem MIC distribution of OXA-48-producing *Enterobacteriaceae*, however, shows a peak at MIC=0.25 mg/L [16]. These isolates will remain undetected if screened by AST only.

When using screening cut-off MICs for CPE detection, which are lower than the susceptibility cut-offs, the sensitivity is still just 80% [17]. Mainly OXA-48-, and some VIM-producers would remain undetected using meropenem screening cut-offs. Since carbapenem-resistant isolates are usually send to reference centers for CPE detection, this may result in an underestimation of true prevalence numbers [18]. In our hospital, we use both culture on screening agars and carbapenemase gene detection directly on rectum samples in patients with a high risk on CPE-carriage to increase the sensitivity of surveillance cultures [19]. Direct screening of rectal swabs for carbapenemases by real-time PCR performed on enrichment broth showed a higher sensitivity than culturing on selective agar plates [20]. However, relying on genotypic tests alone may also be a pitfall. For instance, molecular panels for detection of CPE may have a limited number of carbapenemase gene targets. CPEs that are not detected by the panel may have an evolutionary advantage caused by the limitations of this diagnostic method.

Diagnostic evasion by VRE

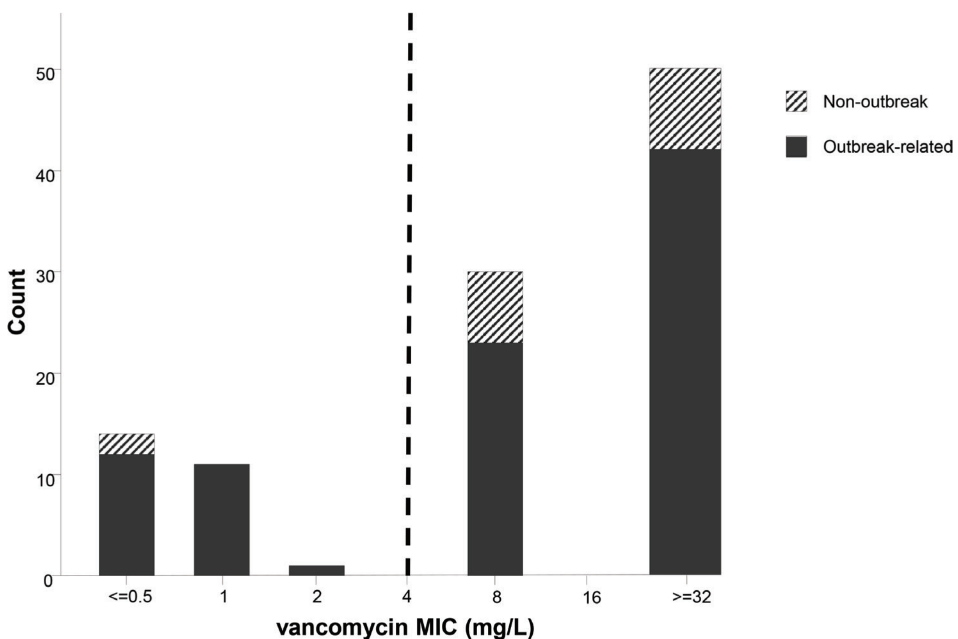
A second example of successful diagnostic evasion by HRMOs is the nationwide emergence of nosocomial outbreaks with vancomycin-resistant *Enterococci* (VRE) in the Netherlands. In the period 2012-2014, 26 outbreaks with VRE have been reported, including reports of local and inter-hospital transmissions [21]. Outbreaks predominantly occurred with VanA- and VanB-type *Enterococcus faecium*, that confer resistance to glycopeptides. *VanB* VRE can easily remain undetected by culturing in routine diagnostics. In addition to the fact that fecal VRE carriage often is detected in very low amounts, vancomycin resistance in *vanB* VRE is not always expressed. These diagnostic challenges have been an important factor in the ongoing transmission of VRE in hospitals in the Netherlands. Several phenotypic screening methods, such as the use of chromogenic agars, have been suggested to identify *vanB* VRE with varying vancomycin MICs [22]. However, VRE suspected colonies growing on Chrome-agars may test vancomycin susceptible in routine AST systems despite positive genotypic confirmation of *vanB*. This could lead to an unnoticed and uncontrolled spread of *vanB* VRE.

In our hospital, patients are screened on a PCR-based method for VRE on admission at the intensive care unit and if patients are transferred from or recently have been admitted in another hospital in the Netherlands or a foreign hospital. If an unexpected VRE case is found, screening is performed in those patients who are at risk of VRE transmission.

We have reviewed VRE data from 2013-2016 in our own hospitals. We searched for all VRE positive patients and selected their first VRE sample. A total of 106 patients were found, all isolates were *vanB E. faecium.* Of these *vanB* VREs, 26 isolates (24.5%) were tested

vancomycin-susceptible by Vitek2 (bioMérieux) according to the EUCAST susceptibility breakpoint of ≤4mg/L [23]. Vancomycin 5 μg paperdisks (Becton Dickenson) were used to phenotypically detect the resistance mechanism, which showed an hazy edge also in the *vanB* positive vancomycin-susceptible isolates. Of these 26 isolates, 24 were outbreak related (92.3%). The two non-outbreak related isolates in the vancomycin-susceptible group were found in a patient transferred from another Dutch hospital and in a patient transferred from a foreign hospital. The other 80 isolates (75.5%) were tested resistant to vancomycin. Of these, 65 isolates (81.3%) were outbreak related (Figure 1). The 15 non-outbreak related isolates in this group were from the surveillance cultures of patients transferred from hospitals abroad (n=1), patients transferred from other Dutch hospitals (n=2), in patients admitted to the ICU (n=8), and in clinical samples (n=4). Noticeably, among these 80 patients with vancomycin-resistant *vanB*, we also detected *vanB* positive vancomycin-susceptible *E. faecium* isolates in follow-up samples from 13 patients. These results are in line with reports in literature. A VRE outbreak in a neonatal ICU in Germany has been reported, in which even 55% of the *vanB* positive VRE isolates were tested vancomycin susceptible [24]. These data show the possible pitfalls in detecting *vanB* VRE in a significant population when only using phenotypic screening tests.

Figure 1: Number of first VRE (all *vanB E. faecium*) isolates from patients during 2013-2016 and their corresponding MIC values. The dashed line represents the vancomycin susceptibly breakpoint of 4mg/L.



Pitfalls in detecting *vanA* VRE have been described due to an altered phenotype of *vanA* VRE. The expression of teicoplanin resistance can be heterogenous conferring into a VanB phenotype [25]. Moreover, isolates can even test vancomycin susceptible due to a silenced *vanA* gene which can easily lead to uncontrolled outbreaks [26, 27].

In a multicenter study the EUCAST disk diffusion method performed significantly better than the Vitek2 system for the detection of clinical enterococci isolates with low and medium level vancomycin resistance [28]. For rapid detection of VRE carriage, diagnostic strategies using selective enrichment broths and molecular detection can be used to increase the sensitivity of diagnostic procedures [29]. Based on above findings, genotypic testing of invasive vancomycin-susceptible enterococci by PCR can be advised. All three diagnostic strategies are being used in our routine diagnostic laboratory.

Diagnostic evasion by MRSA

To detect MRSA carriage, the Dutch laboratory guideline recommends to take samples of the throat, nose, and perineum [1]. Additional body sites should be sampled depending on clinical signs such as wounds, productive cough, skin lesions, or indwelling catheters. To optimize the sensitivity of the cultures, incubation in relatively non-selective enrichment salt-only broths is recommended, followed by culturing for 48 hours on selective MRSA screening agars. Additional rapid molecular test are recommended in case of urgency.

In the Netherlands, patients with risk factors for MRSA–carriage such as recent hospitalization abroad, or *farm workers* at pig *farms*, cattle *farms,* or poultry *farms* are treated upon admission in strict isolation until rapid PCR-based diagnostics are negative. In case of MRSA carriage, patients are treated in isolation and MRSA eradication therapy can be started. This is known as the search and destroy policy [30]. However, PCR-based diagnostics for screening alone would not detect all cases of MRSA-carriage. In a meta-analysis, a sensitivity of 92.5% for the overall pooled PCR estimate has been reported, with a high level of heterogeneity among the studies [31]. PCR-based false negative MRSA results are in our experience usually in patients with a low-level carriage of MRSA. In these cases, culture on chromogenic agar after incubation in broth is more sensitive. In our hospital we use the GeneXpert, an automated PCR-based method to detect MRSA. The lower detection limit for the Xpert MRSA SA nasal assay is about 70 colony forming units (CFU)/sample according to the manufacturer.

A second reason for failure to detect MRSA is that sporadic Staphylococcal Cassette Chromosome mec (SCCmec)-cassette subtypes, which are a common target in commercial

tests, may not be detected by PCR. There is a high diversity in SCCmec-cassettes: already 11 SCCmec-types and numerous subtypes have been designated [32]. The detection of SCCmec by PCR-based tests is still improving, and the coverage has expanded over the recent years. However, since there may be shifts in common lineages, we should be aware of sporadic nosocomial MRSA that may emerge as successful clones, and are undetectable by commercial tests [33]. Variety in the *mecA/mecC* target may also result in failure of MRSA detection by PCR. For instance, MRSAs with the divergent homologue *mecA* ($mecA_{LGA251}$) would not be detected by the Xpert MRSA assays [34].

False-negative PCR results may have a considerable impact in hospitals. Since patients are discharged from strict isolation after negative PCR results, the isolate has an opportunity to spread until the MRSA is identified by culture and the patient is in strict isolation again. To prevent further spread, contact investigations among patients in the same room and health care workers are performed in these cases in the Netherlands. Since PCR-based detection is not reliable in screening for such isolates, the investigation of contacts has to be performed by culture, which delays the time to detection of secondary transmissions.

Not only PCR-based diagnostics, but also culture-based detection may be evaded by MRSA. In 2014, clinical microbiologists were alerted by a report of the monitoring group on an outbreak with a MRSA strain that could easily be missed by routine diagnostics. Although the numbers of transmissions were largely reduced, total control of the outbreak was difficult due to detection problems using conventional culturing. The *mecA*-positive isolate was difficult to culture as the oxacillin MIC was low, ranging from 0.5 to 6.0 µg/mL. Growth on ChromID™ MRSA agar (bioMérieux) plates was strongly inhibited. We tested the outbreak isolate in our own laboratory and found a more then 10-fold decrease in colony numbers if cultured on ChromID™ MRSA plates compared to blood agar, resulting in a detection limit on ChromID™ MRSA below $0.5x10^3$ colonies/100µL. Molecular testing and prolonged subculturing in broths was advised to detect this isolate.

Diagnostic evasion by ESBL

ESBL-detection can be complicated in natural AmpC-producers such as *Citrobacter freundii, Enterobacter* spp., *Hafnia alvei, Morganella morganii, Serratia* spp. and *Providencia* spp, since it mimicks their natural resistance pattern. Antibiotics can select for mutants with derepressed AmpC expression, resulting in resistance to cephalosporins during therapy. Thus, antibiotic treatment with cephalosporins is not recommended [35]. Presence of natural AmpC alone is no condition for HRMO and infection prevention measures.

However, in 2015, several outbreaks in various hospitals in the Netherlands were reported with natural AmpC-producing *Enterobacteriaceae* that acquired additional ESBL genes. This has no consequences for antibiotic therapy choices, however, infection prevention measures need to be taken.

This combined “AmpC-plus-ESBL” phenotype is difficult to distinguish from derepressed-AmpC wild-type resistance. The Dutch laboratory guideline recommend to use cefotaxim and/or ceftazidim to screen for ESBLs with cut-off MIC values for both cephalosporins of >1 mg/L. This screening strategy is also for *Enterobacteriaceae* with natural AmpCs. This leads to many false positive results due to derepressed AmpCs. Phenotypic confirmation based on inhibition ESBL activity by clavulanic acid or cefepime hydrolysis by disk diffusion, Etest or broth microdilution methods is recommended.

Natural AmpC-producing *Enterobacteriaceae* that acquired additional ESBL genes are common in Dutch nosocomial isolates. *Citrobacter freundii* and *Enterobacter cloacae* showed the highest percentages of confirmed ESBL co-producers: 3% of *Citrobacter freundii* (total n=9.432), and 2% of *Enterobacter cloacae* (n=28.027) were recorded by the Dutch national antibiotic resistance surveillance system (ISIS-AR). Microbiologist were explicitly warned for outbreaks with these difficult to detect HRMOs in a report by the monitoring group.

The substantial presence of ESBLs in *Enterobacteriaceae* with natural AmpCs has been underlined in an Asian study [36]. The ESBLs confer additional resistance to fourth generation cephalosporins, compared to the natural broad-spectrum AmpCs. These isolate may represent a hidden reservoir of ESBL-carrying plasmids, which can transfer across species. Numerous outbreaks with ESBL natural AmpC producers have been reported in international literature [37]. Since resistance to 3th generation cephlosporins is very common in natural AmpC producers that do not carry ESBLs [38], the dissemination of ESBL-carrying isolates in hospitals may remain unnoticed.

Implications and future directions

We observed that highly-resistant microorganisms adapt to evade screening strategies. One can consider this process as a prey that evolves to escape from predators. Microbiologists, in their evolutionary role as predators hunting for HRMOs, also have to keep on innovating to update the detection strategies for these micro-organisms that are trying to evade. This may result in an arms race. In evolutionary biology, such an arms race is known as the Red Queens hypothesis [39]. The name of the theory is based on a quote from Lewis Carroll's *Through the Looking-Glass*: “Now, here, *you* see, it takes all the *running you* can do,

to *keep* in the *same* place. If *you* want to *get* somewhere else, *you* must *run* at least twice as *fast* as that!".

To run twice at fast, communication within networks of health care professionals is crucial. In our perspective, we described examples of how Dutch clinical microbiologist were alarmed by a national monitoring group on successful HRMOs that evade routine screening tests. Specific recommendations to adjust diagnostic strategies to detect these pathogens were provided. Additionally, rapid communication within regional networks is of utmost importance. Inter-hospital patient traffic is highest between hospitals in the same regions. As a consequence, hospitals within the same region are at immediate risk of introduction of HRMOs that evade diagnostics and cause outbreaks. We recommend to identify your region of hospitals that are most connected by patient traffic, and set-up communication networks to alarm for difficult to detect HRMO's. Experiences and adjusted diagnostic screening tests should be shared within these networks. Such a regional approach has successfully been applied in the control of MRSA in the Dutch-German cross-border region [40].

We should be aware of the impact of our diagnostics on the introduction and dissemination of resistance elements in our hospitals. The Government of the Netherlands has a national and international mission to combat antimicrobial resistance (AMR). Therefore the NVMM has composed a vision document to maintain the low prevalence of CPE in the Netherlands [41]. By taking CPE as a biological indicator, it is implicitly assumed that other HRMOs will be included in the combat of AMR. To realize the goals, it is of utmost importance that diagnostic methods are continuously innovated and used.

We are aware that optimizing diagnostic screening will increase costs. On the other hand, our examples have shown that failure of detection by routine diagnostics may lead to uncontrolled outbreaks. These outbreaks can lead to enormous financial expenses; costs may rise up to €1,369 per patient per day [42]. Moreover, detection of HRMO carriage allows for directed antibiotic treatment of patients developing infections by these HRMOs.

Cost reductions in innovation of diagnostics for screening purposes are foretold to result in nosocomial outbreaks with HRMOs evading our screenings methods. We would be outsmarted by prokaryotes.

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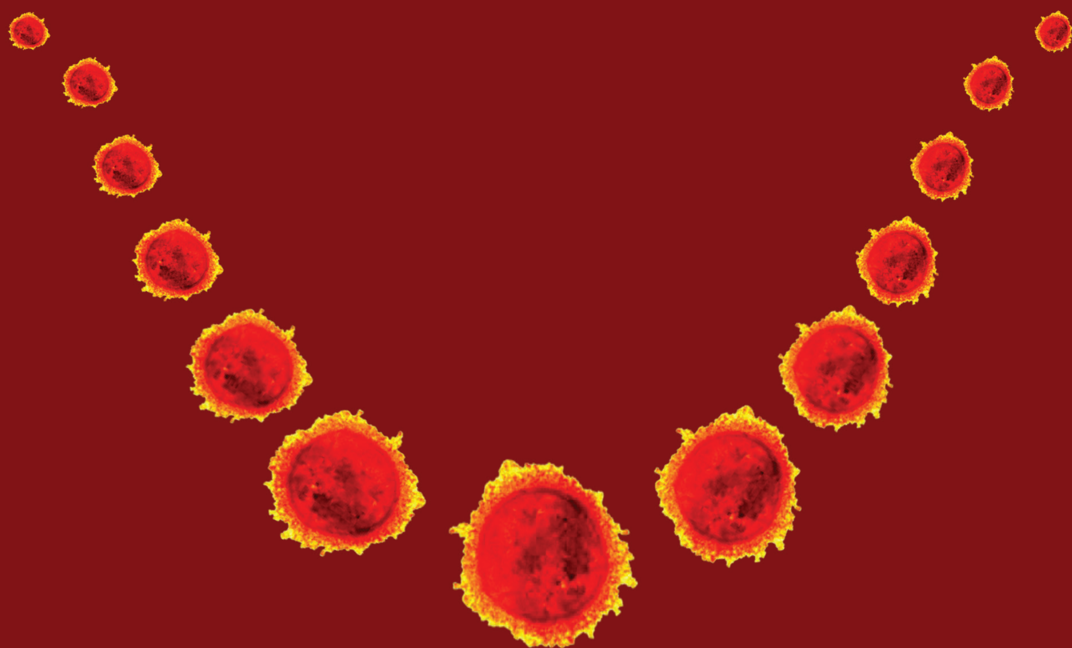

Elucidating vancomycin-*resistant Enterococcus faecium* outbreaks: the role of clonal spread and movement of mobile genetic elements.

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Short title: Transposons on the move in VREfm outbreaks

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ABSTRACT

Introduction:

Vancomycin resistant *Enterococcus faecium* (VREfm) has emerged as a nosocomial pathogen worldwide. The dissemination of VREfm is due to both clonal spread and spread of mobile genetic elements (MGEs) such as transposons. We aimed to combine *vanB*-carrying transposons characteristics with core-genome MLST (cgMLST) typing and epidemiological data to understand the pathways of transmission in nosocomial outbreaks.

Methods:

Retrospectively, 36 VREfm isolates obtained from 34 patients from seven VREfm outbreak investigations in 2014 were analysed. Isolates were sequenced on a MiSeq and a MinION instrument. *De novo* assembly was performed in CLC Genomics Workbench, the hybrid assemblies were obtained through Unicycler v0.4.1. Ridom SeqSphere+ was used to extract MLST and cgMLST data. Detailed analysis of each transposon and their integration points were performed using Artemis Comparison Tool (ACT) and multiple blast analyses.

Results

Four different *vanB* transposons were found among the isolates. CgMLST divided ST80 isolates into three CTs; CT16, CT104 and CT106. ST117 isolates were divided into CT24, CT103 and CT105. Within VREfm isolates belonging to CT103, two different *vanB* transposons were found. In contrast, VREfm isolates belonging to CT104 and CT106 harboured an identical *vanB* transposon.

Conclusion:

CgMLST provides a high discriminatory power for the epidemiological analysis of VREfm. However, additional transposon analysis is needed to detect horizontal gene transfer. Combining these two methods allows to investigate both clonal spread as well as the spread of MGEs. This leads to new insights and thereby better understanding of the complex transmission routes in VREfm outbreaks.

INTRODUCTION

Enterococcus faecium has emerged as a nosocomial pathogen worldwide. Vancomycin resistant *E. faecium* (VREfm) outbreaks are mainly caused by successful hospital-associated (HA) *E. faecium* isolates that acquired the *vanA* or *vanB* gene [1]. The dissemination of VREfm is the result of both clonal spread of successful clones, mainly ST17, ST18 and ST78 [2] and the exchange of mobile genetic elements (MGEs) such as chromosomal fragments [3] and plasmids [1, 4, 5]. The *vanA* gene is part of an operon of seven genes, carried by the Tn*1546* transposon, which can be located on various plasmid types or can be integrated into the chromosome [6, 7]. Similarly to *vanA*, *vanB* is also a part of an operon that consists of seven genes, generally located on the conjugative transposon Tn*1549*/Tn*5382*. Like *Tn1546*, this transposon can also be located on various types of plasmids or can be integrated into the chromosome [1, 4].

In our hospital, we mainly find *vanB* VREfm. Successful HA vancomycin susceptible *E. faecium* (VSEfm) lineages may acquire the *vanB* gene by different pathways. It can occur by *de novo* acquisition of *Tn1549* from anaerobic gut microbiota [8]. Another mechanism is through the exchange of large chromosomal fragments, including Tn*1549*, between *vanB* VREfm and VSEfm [3].

In outbreak investigations, rapid and accurate typing is required to investigate the genetic relatedness between patients' isolates. This information is essential to demonstrate nosocomial transmission. Till 2014, most of VREfm isolates in our hospital were typed by multi-locus variable-number tandem repeat analysis (MLVA). MLVA is an easy, fast and highly reproducible method to type VREfm [9], but not discriminatory enough in outbreak investigations. MLST is a key tool to study the genetic relatedness and epidemiology of *E. faecium* isolates [10]. However, the discriminatory power of MLST is also insufficient in nosocomial outbreak investigations [11]. In addition to the inferior discriminatory power, MLST-based typing may be unreliable due to recombination events in the MLST loci, which can cause a high number of discrepancies between WGS based typing and MLST [8, 12, 13].

In 2014, WGS was implemented in our laboratory for outbreak investigations of multi-drug resistant microorganisms, including VREfm [14]. The challenge of using WGS is to rapidly analyse and interpret the relevant information [15, 16]. In 2015, a core genome (cg)MLST scheme (consisting 1.423 target genes) for *E. faecium* was developed [17]. This gene-by-gene typing based approach uses a defined set of genes to extract an allele-based profile which makes it scalable and comparable between laboratories. However, cgMLST may also be misleading if horizontal transfer of a single *vanB*-carrying transposon occurs between different *E. faecium* clones during a VREfm outbreak event.

In this study, we retrospectively analysed available draft genome sequences of VREfm isolates from several outbreaks in 2014 in our region and investigated relevant epidemiological data. Next, a detailed characterisation of *vanB*-carrying transposons was performed to determine possible horizontal gene transfer. Hereby spread by clonal expansion as well as by horizontal gene transfer are studied.

METHODS

Study population and infection control protocols

We retrospectively analysed VREfm outbreaks that occurred in the University Medical Center Groningen, the Netherlands in 2014. In 2014, 75 new patients with VREfm were detected. Microbiological data and infection records were used. Infection records included epidemiological information about positive VRE patients. Epidemiological data included dates of when patients were found to be positive, ward and room numbers, patient transfer data and microbiological typing data. We also made use of an epidemiological program to visualize and analyze patient transfers in more detail over several wards and rooms in time by using bed occupancy databases. Herein multiple patients and wards could be included. From 2014 on, concurrent VRE outbreaks have arisen, as experienced by many hospitals in the Netherlands.

By protocol, we screen the following patients for VRE upon admission: patients who have been admitted in a hospital abroad within the past year; patients directly transferred from another hospital in the Netherlands; patients who are admitted to the intensive care and haematology wards; and adopted children. In the Netherlands, it is recommended to screen adopted children for MRSA, as they are frequently from countries that are highly endemic for MRSA. We have chosen to extend the screening in adopted children, by screening for all highly-resistant microorganisms (HRMOs), including VRE. Patients previously known to carry VRE of which the last positive VRE culture was less than one year ago, are treated in contact isolation and additional rectal swabs are taken for VRE screening. At least five rectal swabs are needed to discard the isolation measures in VRE positive patients and those that were known to carry VRE less than one year ago. In patients previously known to carry VRE more than more year ago are treated in contact isolation, unless one of more negative previous VRE cultures were registered. Additional one rectal swab is taken for VRE screening. If this is negative, isolation measures can be discarded. Patients carrying VRE are treated in contact isolation in a single room, using a disposable gown and gloves by the

personnel. Screening of contact patients is performed if there has been exposure of other patients in the same room, or if nosocomial acquisition of VRE is suspected. Since not all patients in our hospital are routinely screened, nosocomial acquisition (e.g. >48 hours) is difficult to define. However, in cases of VRE positive patients who were previously screened VRE negative and in situations of ongoing VRE spread, this is considered as nosocomial acquisition. Screening of contact patients is performed as follows: first, (ex-) roommates of the VRE-positive patient will be screened. If there are one or more VRE-positive contact patients, all patients at the ward and if relevant, ex-patients that have stayed in the affected ward will be screened. The screening is repeated until no new positive VRE patients are detected in at least three rounds of screening, whereas at least 48 hours between each screening round is required. On average, the last screening round will be seven days after (possible) exposure since transmission and subsequent rectal colonization takes time [18].

VRE culturing

VRE culturing was preceded by PCR-screening as described previously [19]. In short, rectal swabs were inoculated in enrichment broth. After 24 hours incubation, a *vanA/vanB* PCR (Xpert®vanA/vanB, Cepheid) was performed on a GeneXpert® XVI (Cepheid) and when positive, the broth was subcultured on VRE Brilliance agars (Oxoid®). Agars were incubated for 24-48 hours and identification and antibiotic susceptibility testing were performed on suspected colonies by MALDI-TOF Mass Spectrometry (Bruker) and VITEK®2 (Biomerieux), respectively. Additionally, we used vancomycin disk diffusion since this method is more sensitive in detecting enterococci isolates with low- and medium-level vanB-type vancomycin resistance [20]. Moreover, identified *E. faecium* isolates were again genotypically tested for the presence of *vanA* and *vanB* genes by PCR using the Xpert®vanA/vanB assay.

Standard, all first VREfm isolates of each patient were typed by MLVA, according to the method described by Top *et al.* [9]. In some cases, e.g. patients that were infected as well as colonized by VRE or harbouring *vanA* as well as *vanB* VRE, multiple VRE isolates were typed. In 2014, we started to implement WGS for VREfm outbreak investigations. In this implementation phase, only a representative subset of isolates that were typed by MLVA were selected for WGS and typed by cgMLST.

WGS and typing methods

Genomic DNA was extracted using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, US) following the manufacturer instructions. The DNA concentration and

purity were measured by the Qubit dsDNA HS and BR assay kit (Life technologies, Carlsbad, CA, US). A DNA library was prepared using the Nextera XT v2 kit (Illumina, San Diego, CA, USA) and then run on a MiSeq sequencer (Illumina) for generating paired-end 250-bp reads. De novo assembly was performed by CLC Genomics Workbench v7.0.4 (QIAGEN, Hilden, Germany) after quality trimming (Qs ≥ 20) with optimal word sizes. All procedures were performed as previously described [21]. For the long-read sequencing, libraries of samples A13, A16, A20 and A22 were prepared without shearing to maximize sequencing read length. Samples were barcoded with the Native Barcoding Kit 1D (EXP-NBD103) and libraries were prepared using the Ligation Sequencing Kit 1D (SQK-LSK108). The library was loaded onto an FLO-MIN106 R9.4 flow cell and ran on a MinION device (48 hours). Base calling was performed using Albacore v1.2.2. Data quality was analyzed through Poretools v0.6.0. [22]. Hybrid assemblies were performed using Unicycler v0.4.1. [23]. Bandage v0.8.1 [24] was used to visualize the assembly graphics. Genes of interest were detected using ResFinder.

MLST STs and cgMLST CTs were extracted from the draft genomic sequences using SeqSphere+ version 3.0.1 (Ridom GmbH, Münster, Germany). For the cgMLST analysis, Seqsphere+ used the *E. faecium* scheme published previously [17], considering a cluster alert distance of 20 different alleles. The *vanB*-carrying transposons were identified by BLAST comparisons of *de novo* and hybrid assemblies with the reference sequence of *Tn1549* (GenBank AF192329.1) using the Webact online tool (http://www.webact.org/WebACT/home), [25] under default settings. Detailed analysis of each transposon as well as their integration points were performed using ACT [26] and multiple blast analyses.

Ethics:

The bacterial isolates used for the present analyses were collected in the course of routine diagnostics and infection prevention control. Oral consent for the use of such clinical samples for research purposes is routinely obtained upon patient admission to the UMCG, in accordance with the guidelines of the Medical Ethics Committee of the University Medical Center Groningen. All experiments were performed in accordance with the guidelines of the Declaration of Helsinki and the institutional regulations, and all samples were anonymized.

Nucleotide sequence accession numbers

Sequence data obtained in this study has been deposited at the European Nucleotide Archive (ENA) under BioProject no. PRJEB25590. The hybrid assemblies have been deposited in NCBI under BioProject no PRJNA477347.

RESULTS

Description of outbreak clusters based on epidemiological data

During the implementation period of WGS, 36 representative isolates of 34 patients were sequenced and their draft genome sequences were available for analysis. Based on epidemiological data from infection prevention records, these 34 patients were involved in six outbreak episodes in 7 different wards in 2014. All first VRE isolates of individual cases were assessed, except for two patients, from whom multiple isolates were selected for sequencing (A4 & A4.1 and A22 & A22.1).

Details of the isolates and to which outbreak investigation they belonged are presented in Table 1. Initial outbreak investigations were performed using epidemiological information as described in the methods. Outbreak investigation A took place in April 2014 on ward 1 and 12 patients were involved. Of 10 of these patients, the genome sequences of the obtained isolates (A1 and A4-A13, including A4.1) were available. One of the patients admitted to ward 1 was previously hospitalized in another hospital located in the region. Two isolates (A2 and A3) were therefore obtained from possible contact patients from the other regional hospital and were included in this analysis. Outbreak investigation B took place in July 2014 on ward 1 and 4 patients were involved. Of two patients, the genome sequences of the obtained isolates (A14 and A15) were available. Outbreak investigation C took place in July 2014 on ward 5 and 6 and 10 patients were involved. Of 5 of these patients, genome sequences of the obtained isolates (A16-A20) were available. According to epidemiological data outbreak investigation D took place in November 2014 on ward 7 and involved in total 11 patients. Of 8 of these patients, the genome sequences of the obtained isolates (A21-A28) were available. Also in November, outbreak investigation E took place on ward 2, involving 11 patients. Of 3 of these patients, the genome sequences of the obtained isolates (A29-A31) were available. Finally, outbreak investigation F took place in December 2014 on several wards, involving 7 patients. Of 3 of these patients, the genome sequences of the obtained isolates (A32-A34) were available from a selected ward (ward 4).

Patients A22 and A27 were colonised with *E. faecium* isolates carrying both the *vanA* as well as the *vanB* gene. The *vanA* gene resided on the chromosome, while the *vanB* gene was located on a plasmid. This study will further focus on the *vanB* VREfm and Tn*1549*/Tn*5382* transposon analysis since the rest of the patients were colonised with only *vanB* VREfm.

Table 1: Epidemiological and molecular data of the 36 isolates from 34 patients used in this study.

Sample ID	Outbreak cluster	Month	Ward(s)	Age	Gender	VRE type	Vancomycin MIC mg/L	Isolation source	MT[a] type	ST[b] type	CT[c] type	Sample date	Location *vanB* gene	Transposon type
A1	A	April	Ward 1	43	M	*vanB*	8	Rectal swab	12	117	24	8/3/2014	Chromosome	1
A2	A	April	*	73	M	*vanB*	8	Rectal swab	12	117	24	16/5/2014	Chromosome	1
À3	A	April	*	76	M	*vanB*	8	Sputum	12	117	24	19/5/2014	Chromosome	1
A4	A	April	Ward 1	65	V	*vanB*	>=32	Bile	12	117	24	1/4/2014	Chromosome	1
A4.1	A	April	Ward 1	65	V	*vanB*	>=32	Rectal swab	12	117	103	13/5/2014	Chromosome	1
A5	A	April	Ward 1	67	V	*vanB*	>=32	Rectal swab	12	117	24	16/4/2014	Chromosome	1
A6	A	April	Ward 1	69	V	*vanB*	>=32	Rectal swab	12	117	24	16/4/2014	Chromosome	1
A7	A	April	Ward 1	59	M	*vanB*	>=32	Rectal swab	12	117	24	21/4/2014	Chromosome	1
A8	A	April	Ward 1	82	V	*vanB*	8	Rectal swab	12	117	24	24/4/2014	Chromosome	1
A9	A	April	Ward 1	59	M	*vanB*	8	Rectal swab	12	80	16	29/4/2014	ND[d]	ND
A10	A	April	Ward 1	69	V	*vanB*	8	Rectal swab	12	117	24	1/5/2014	Chromosome	1
A11	A	April	Ward 1	67	M	*vanB*	>=32	Rectal swab	12	117	24	4/5/2014	Chromosome	1
A12	A	April	Ward 1	77	M	*vanB*	8	Rectal swab	12	117	24	2/4/2014	Chromosome	1
A13	A	April	Ward 1	61	V	*vanB*	>=32	Rectal swab	12	117	24	29/4/2014	Plasmid	2
A14	B	July	Ward 1	61	M	*vanB*	<=0.5	Rectal swab	144	262	60	29/6/2014	ND	ND
A15	B	July	Ward 1	78	M	*vanB*	8	Rectal swab	12	117	103	1/7/2014	Plasmid	2
A16	C	July	Ward 5&6	58	M	*vanB*	>=32	Rectal swab	1	80	104	22/6/2014	Plasmid	4
A17	C	July	Ward 5&6	54	M	*vanB*	8	Rectal swab	1	80	104	28/6/2014	Plasmid	4
A18	C	July	Ward 5&6	49	M	*vanB*	8	Faeces	12	117	103	30/6/2014	Plasmid	2
A19	C	July	Ward 5&6	65	V	*vanB*	>=32	Rectal swab	12	117	103	25/7/2014	Plasmid	2
A20	C	July	Ward 5&6	61	M	*vanB*	<=0.5	Rectal swab	12	117	105	3/11/2014	Chromosome	3
A21	D	November	Ward 7	68	V	*vanB*	>=32	Rectal swab	1	80	104	29/10/2014	Plasmid	4

Sample ID	Outbreak cluster	Month	Ward(s)	Age	Gender	VRE type	Vancomycin MIC mg/L	Isolation source	MT[a] type	ST[b] type	CT[c] type	Sample date	Location *vanB* gene	Transposon type
A22	D	November	Ward 7	62	M	*vanA* and *vanB*	>=32	Faeces	1	80	106	31/10/2014	Plasmid	4
A22.1	D	November	Ward 7	62	M	*vanA* and *vanB*	>=32	Rectal swab	1	80	106	4/11/2014	Plasmid	4
A23	D	November	Ward 7	66	V	*vanB*	8	Rectal swab	1	80	104	4/11/2014	Plasmid	4
A24	D	November	Ward 7	66	V	*vanB*	1	Rectal swab	1	80	104	4/11/2014	Plasmid	4
A25	D	November	Ward 7	70	M	*vanB*	1	Rectal swab	1	80	104	4/11/2014	Plasmid	4
A26	D	November	Ward 7	59	M	*vanB*	8	Rectal swab	1	80	104	4/11/2014	Plasmid	4
A27	D	November	Ward 7	50	M	*vanA* and *vanB*	>=32	Rectal swab	1	80	106	18/11/2014	Plasmid	4
A28	D	November	Ward 7	56	V	*vanB*	8	Rectal swab	1	80	104	19/11/2014	Plasmid	4
A29	E	November	Ward 2	57	M	*vanB*	>=32	Rectal swab	12	117	103	1/12/2014	Plasmid	2
A30	E	November	Ward 2	66	M	*vanB*	>=32	Rectal swab	12	117	103	2/12/2014	Plasmid	2
A31	E	November	Ward 2	60	V	*vanB*	8	Rectal swab	12	117	103	16/12/2014	Plasmid	2
A32	F	December	Ward 4	64	M	*vanB*	>=32	Rectal swab	12	117	103	22/12/2014	Plasmid	2
A33	F	December	Ward 4	69	M	*vanB*	>=32	Rectal swab	12	117	103	28/12/2014	Plasmid	2
A34	F	December	Ward 4	87	M	*vanB*	>=32	Rectal swab	12	117	103	31/12/2014	Plasmid	2

a MT type=MLVA type, b ST type = sequence type, c CT=cluster type. d ND=not determined *=these isolates were genetically related to outbreak A, but were obtained from patients from a regional hospital.

Discrepancies between epidemiological links and typing results

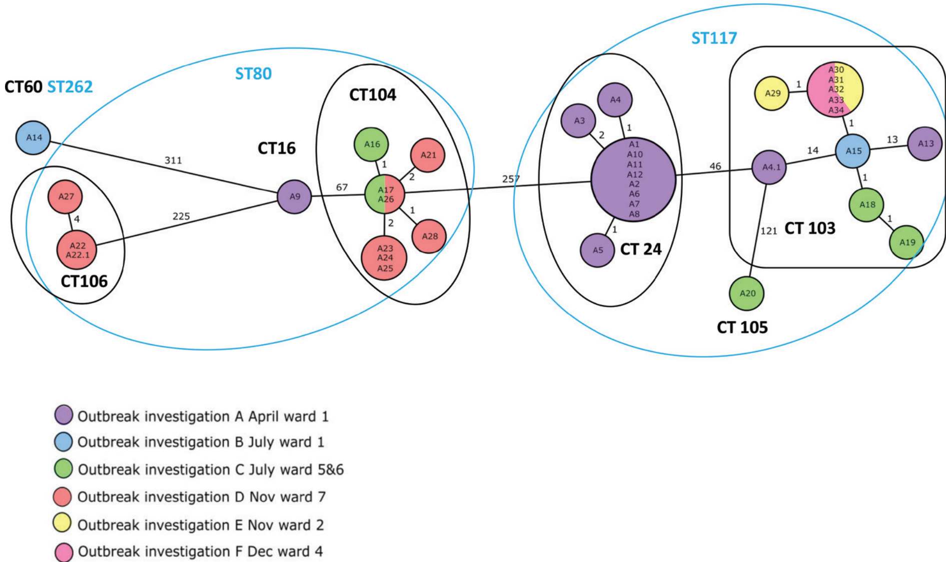
Initial MLVA typing showed three MLVA types (MT); MT1 (n=12), MT12 (n=23) and MT144 (n=1) (Table 1). Based on MLST typing, the isolates belonged to ST80 (n=12), ST117 (n=23) and ST262 (n=1). The clusters based on MLVA, and MLST matched except for isolate A9. CgMLST typing identified seven different clusters: CT103 (n=11), CT24 (n=11), CT104 (n=8), CT105 (n=1), CT106 (n=3), CT60 (n=1) and CT16 (n=1) (Table 1). The minimum spanning tree of the cgMLST typing results of the 36 sequenced isolates is shown in Figure 1.

In outbreak investigation A, the typing results of MLVA, MLST and cgMLST confirmed that 11 out of the 14 isolates were genetically related. These isolates belonged to CT24 whereas the isolates A13 and A9 were representing CT103 and CT16, respectively. Isolate A4.1 belonging to patient A4 of whom two isolates were sequenced, is discussed below. Patient A13 was initially considered as the index patient of the outbreak investigation A, because the patient was known to be colonized with VREfm already in March 2013. However, patient A13 was associated with another outbreak investigation which is discussed below. Based on the cgMLST results, patient A1 was eventually found to be most likely the index patient of the outbreak. As mentioned earlier, this patient was transferred from another regional hospital. Interestingly, the isolates of the three patients from the regional hospital (A1-A3), clustered together with the isolates (A4-A8 and A10-12) obtained from eight patients in our hospital. Isolate A9 belonged to CT16 and eventually could not be linked with any of the outbreaks. The two isolates from outbreak investigation B were totally different based on MLVA, MLST and cgMLST. In case of outbreak investigation C, MLST showed two isolates belonging to ST80 and three isolates belonging to ST117. The cgMLST results identified the presence of three CTs among the isolates in this outbreak investigation; CT103, CT104 and CT105. By MLVA and MLST typing isolates of outbreak investigation D could not be discriminated, but cgMLST divided them into two distinct clusters: five isolates belonged to CT104, and three to CT106. The isolates of CT106 were *vanA*/*vanB* co-producers. Based on cgMLST, the three isolates from outbreak investigation E belonged to CT103 as well as the three isolates from outbreak investigation F.

vanB-carrying transposons characterisation

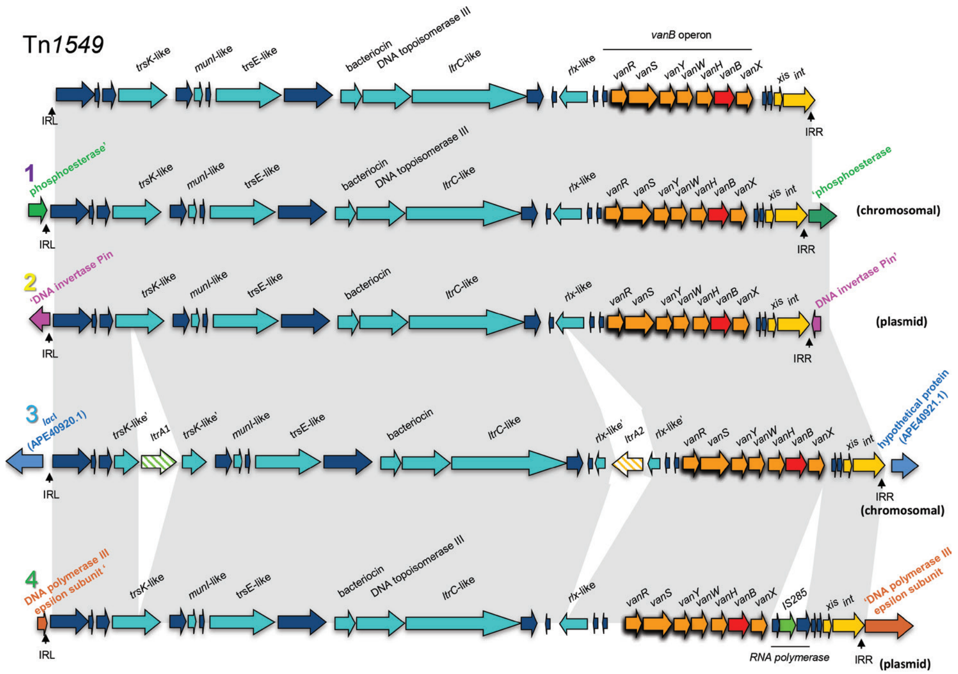
Based on the *de novo* assemblies and the hybrid assemblies generated from sequencing data of the 36 VREfm isolates, the *vanB*-carrying transposons and the genomic locations of these MGEs were investigated in more detail. Unfortunately, isolates A9 and A14 lost the *vanB* gene and were therefore excluded from this analysis. Four different transposons carrying the *vanB* operons were detected, further referred to as transposon type 1, 2, 3 and 4 (Figure 2).

Figure 1: Minimum spanning tree based on cgMLST (1,423 target genes). The different colors indicate the six different outbreaks investigations based on epidemiological data. Numbers indicate patients. Of patients 4 and 22, two samples were included, indicated as samples A4 and A4.1 and samples A22 and A22.1, respectively. The numbers next to the lines corresponds to allele differences between the isolates. ST = sequence type (blue); CT = cluster type (black).



Transposon type 1 was detected in all 13 VREfm isolates belonging to CT24 (A1-A8 and A10-12) and in one isolate belonging to CT103 (A4.1). The overall DNA sequence of this transposon was similar to the previously described transposon Tn*1549*/Tn*5382* (GenBank: AF192329.1) with 99 SNPs difference. In all 14 isolates, the identical *vanB* transposon was located on the bacterial chromosome integrated into the phosphoesterase gene (Genbank locus_taq: BO233_04565). Interestingly, isolates A4 with CT24 and A4.1 with CT103 were obtained from the same patient and both carried transposon type 1. In total, six isolates from rectum and bile were collected from patient A4 in the period from April till October 2014. We decided to sequence these additional six strains to verify this observation. Indeed, two isolates from rectum (A4.1 and 4.2) belonged to CT103. Two isolates from rectum (A4.3 and A4.4) and two from bile (A4 and A4.5) belonged to CT24. Details are shown in supplementary Table S1. Again, all six VREfm isolates harboured the identical *vanB* transposon (Transposon type 1) with identical insertion sites.

Figure 2: The four different *vanB* transposons in comparison to the reference *Tn1549*. Transposons were numbered as in Figure 4. All transposons have their unique insertion sites into different genes as indicated on both sides. Transposons 1 and 3 are located on the chromosome, whereas transposons 2 and 4 on the plasmids, as indicated in the Figure. IRL=left inverted repeat; IRR=right inverted repeat.



Transposon type 2 was detected in 10 isolates belonging to CT103 (A13, A15, A18, A19 and A29-A34). This transposon was found to be integrated into the plasmid DNA invertase Pin gene (Genbank locus_taq: BO233_15550). The overall DNA sequence of this transposon shared the lowest similarity in comparison with the reference Tn*1549*/Tn*5382* transposon and differed by 261 SNPs.

Transposon type 3 was detected in the single isolate of CT105 (A20). The transposon was located on the bacterial chromosome integrated between two genes; *lacI* (Genbank locus_taq :BO233_10750) and a gene encoding a hypothetical protein (GenBank locus_taq: BO233_10755). This transposon was similar to the reference Tn*1549*/Tn*5382* transposon, differing by 100 SNPs. In this transposon, two not previously reported regions were detected. A region of 2677 bp in size, was integrated into the gene encoding a *trsK*-like protein and contained a gene encoding an RNA-directed DNA polymerase sharing an 99% amino acid similarity with *Clostridioides difficile (*NCBI Reference Sequence: WP_044491975.1) The

second region of 2434 bp in size was integrated into a *Rlx* like protein and contained a gene probably responsible for encoding a group II intron reverse transcriptase/maturase. Interestingly, protein blast analysis revealed a substantial, 97% amino acid similarity, with a new identified protein homologous to a protein present in *Faecalibacterium spp*. (NCBI Reference Sequence: WP_087366583.1).

Transposon type 4 was detected in all CT104 (n=8) and CT106 (n=3) isolates. This transposon was located on a plasmid and integrated into the DNA polymerase III epsilon subunit gene. The transposon differed by 81 SNPs from the reference transposon and contained a novel insertion sequence IS*285* present downstream of *vanX*. This insertion sequence is related to *Ruminococcus* spp. as there was 98% amino acid identity with the IS*256* family transposase of *Ruminococcaceae bacterium* cv2 (NCBI Reference Sequence: WP_055079492.1).

Combining epidemiological data, cgMLST, and transposon characterisation

The analysis by cgMLST of all isolates showed clustering based on genetic relatedness of isolates which were initially grouped into different outbreak events. Isolates within CT103 belonged to outbreak clusters A, B, C, E, and F, but clustered together based on cgMLST. In addition, the identical Type 2 transposon was detected in VREfm from 10 patients, that were previously grouped into different outbreak clusters B, C, E, F. To elucidate this observation, we attempted a more detailed analysis by combining epidemiological data and to visualise patients transfer data and bed occupancies in our epidemiological program, as well as cgMLST and transposon analysis. Figure 3 shows the transfers/movements of 8 patients within and between four different hospital wards over time that were found to carry VREfm with the identical Type 2 transposon. By this approach, we identified overlaps in time and wards linking the patients A13, A15 and A29 till 34. No direct epidemiological links were found between patients A18 and A19 comparing to the other patients carrying VREfm with the Type 2 transposon.

Taking all the results together it was concluded that most likely three VREfm outbreaks took place (Figure 4). The first outbreak was caused by isolates of CT24 carrying transposon Type 1, including a case of within-patient transfer (patient A4) to CT103. A second outbreak was caused by isolates belonging to CT103 with transposon Type 2. The third outbreak was associated with isolates of CT104 and CT106 connected by horizontal transfer of transposon Type 4. All other isolates represented individual cases.

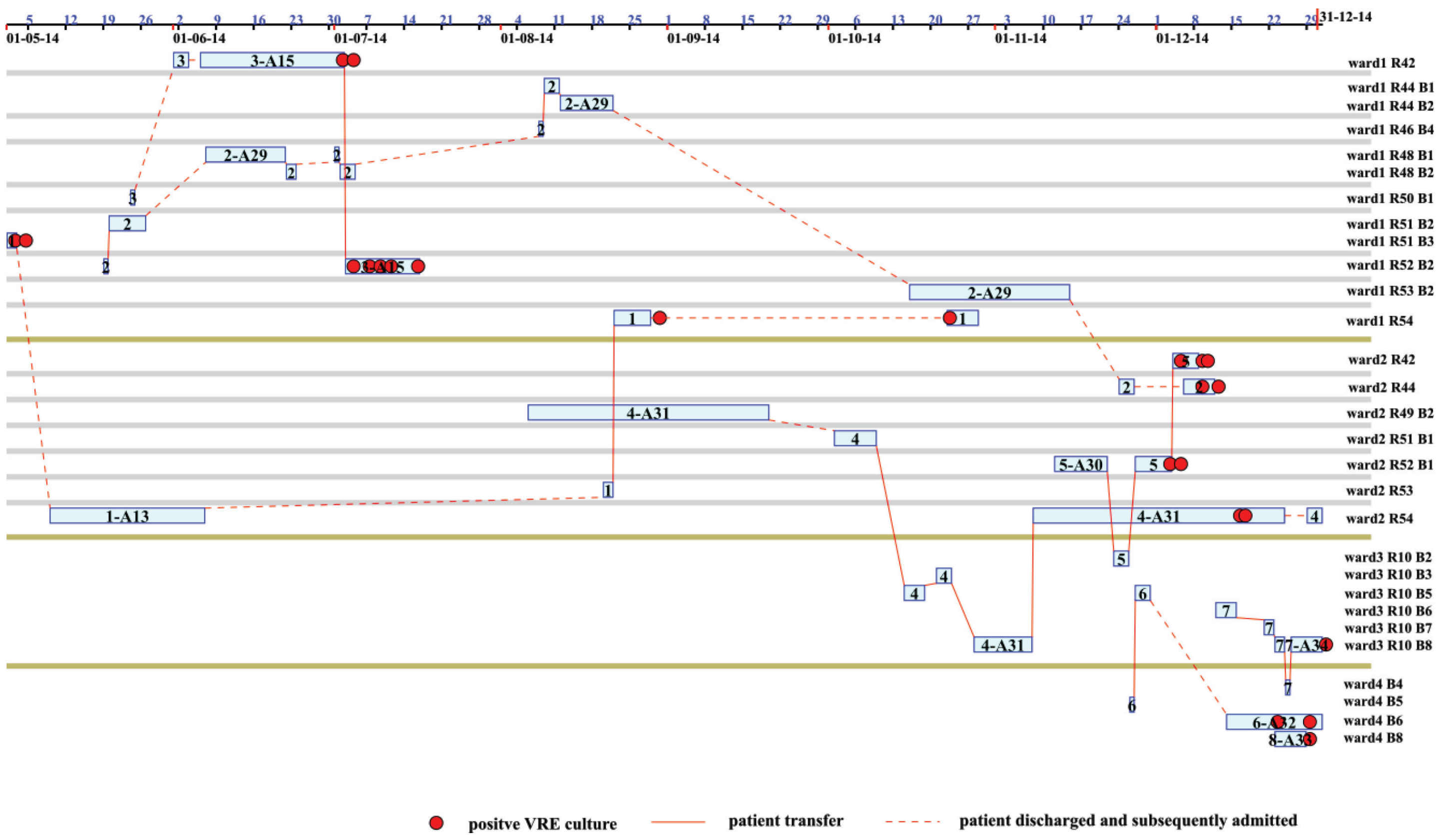
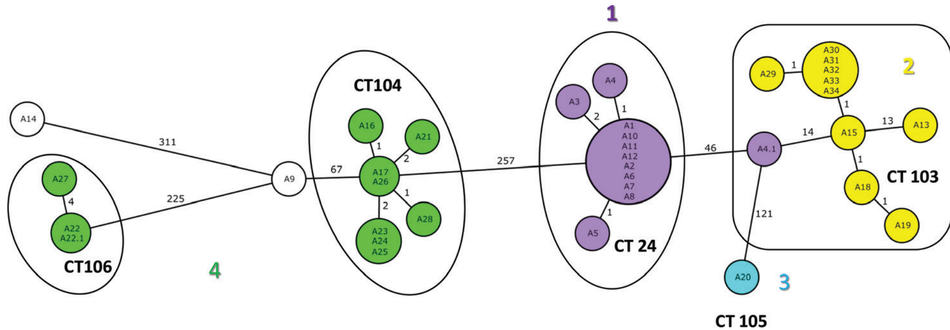


Figure 3: Patient movements among four different wards during the period from May until the end of December 2014**.** The figure shows the movements of patients A13, A15 and A29-A34. The numbers indicate the patients: 1=A13; 2=A29; 3=A15; 4=A31; 5=A30; 6=A32; 7=A34; 8=A33. On the right, the 4 different wards including the different room numbers (R) and beds (B) are shown.

Figure 4: Minimum spanning tree based on cgMLST (1,423 target genes). In contrast to Figure 1, colours now indicate the four different *vanB* transposon types (numbered in bold, 1-4). Isolates from A9 and A14 were excluded due to the loss of the *vanB* gene. From patient A4 and patient A22 two samples were included in the analysis (samples A4&A4.1 and A22&22.1 respectively). The numbers next to the lines correspond to allele differences between the isolates. ST=sequence type (blue), CT=cluster type (black).



DISCUSSION:

In this study, WGS and epidemiological data obtained from VREfm isolates during outbreaks in 2014 in our region were retrospectively analysed. Characterisation of *vanB*-carrying transposons in VREfm isolates was shown to be an additional value in the outbreak investigation. Transposon analysis is essential in cases where outbreaks are caused by the movement of particular MGEs. The horizontal transfer of *vanB*-carrying transposons was identified in two outbreak events. First, it was shown to occur within an individual patient, in whom isolates belonging to different clusters contained an identical transposon. Second, patients from outbreak investigation D belonging to different CTs (CT104 and CT106) carried VREfm isolates harbouring the same transposon. Thus, this study clearly shows the importance of *vanB* transposon investigation. VREfm isolates belonging to identical CTs defined by cgMLST, can acquire different *vanB* carrying transposons *de novo*, which can be incorrectly interpreted based on cgMLST only. Although this situation only occurred in one patient in our study, this phenomenon has been described [8, 27] and we hypothesize that this will happen more often if VREfm outbreaks analysis also include transposon investigation. On the contrary, VREfm isolates belonging to different CTs can also harbour the same *vanB* transposon and thereby belonging to the same outbreak cluster. Other studies have also explicitly shown the importance of transferable MGEs in VREfm outbreaks [4, 8, 27, 28]. Molecular typing methods

such as MLVA and MLST are used in the analysis of VREfm outbreaks and for epidemiological surveillance [11, 29-32]. However, these methods only allow to investigate clonal spread, as is also the case with cgMLST alone. These methods will fail in case the outbreaks are further complicated by horizontal gene transfer of MGE, like plasmids and/or transposons.

We observed the presence of the same *vanB* transposon in VREfm isolates belonging to distinct lineages, showing exchange of genomic material between VREfm and VSEfm. We also found transposons with low DNA sequence homology indicating that they originated from other species and the presence of insertion sequences originating from anaerobic bacteria which indicates transposon acquisition from anaerobic gut microbiotica to VSEfm. The occurrence of these two events are both important factors in the emergence of (*vanB*) VREfm.

In addition to the detection of horizontal gene transfer, this study shows that transposon-analysis even increases the discriminatory power of WGS compared to only using the data for cgMLST. On the other hand, cgMLST provides a higher discriminatory power than MLVA and MLST typing only. It is able to distinguish genetically closely related isolates even if they belong to the same ST lineage. This was the case for ST80 and ST117 in our study, each divided into three different CTs. Both ST117 and ST80 are frequently found in hospitals and associated with outbreaks [33-38] and typically belong the hospital associated clade A [39, 40]. CgMLST analysis also allows inter-laboratory exchange of typing data. This is important as the exchange of patients between hospitals and hospital units can contribute to the spread of VREfm within the healthcare networks. Indeed, using cgMLST allowed us not only to show clonal spread within our own hospital, but also intraregional spread via a connected hospital in our healthcare region. Recent studies from Denmark and England, where WGS for VREfm isolates was used as well, have also shown VREfm transmission within a healthcare network [11, 27, 41]. Therefore, it is wise to set up a local healthcare network surveillance program by identifying healthcare facilities that are most connected by patient traffic to allow optimal regional infection prevention measures. Such networks are currently recommended by the Ministry of Health, Wellbeing and Sports in the Netherlands, and is already well established in our Healthcare region [42].

Collecting epidemiological information is crucial to understand the transmission pathways during an outbreak [30, 43]. However, patients transfer can be quite complicated to follow as is shown in our study. Although an epidemiological link could be found for the majority of patients included in this study, some of the transmission pathways were still not fully understood. This could partially be explained by the fact that we were not able to

sequence all VREfm isolates present in all patients involved in the outbreaks investigations during the implementation of WGS in 2014. Moreover, data was not always directly available. Nowadays, WGS is fully implemented as a standardized typing methods for VRE in our institute and we have speed up the turnaround time to 48 hours (from culture to WGS data). Ideally, all WGS data should not only be used for cgMLST typing, but also in parallel for transposon analysis. Preferably, to create a complete picture of the outbreaks, all VREfm positive patients should be sequenced and included in the cgMLST analysis. Indeed, based on these preliminary results, we have now implemented WGS for every new VREfm isolate per patient. Because of horizontal gene transfer, it should also be considered to include several/all VREfm isolates per patient in outbreak investigations. This can lead to a further increase in the already enormous costs of outbreak investigations. However, advances in sequencing technologies and analysis tools, increases the output, speeds up the analysis and reduces the costs of WGS and by allowing for more focused infection control measures reducing probably overall costs [14, 15, 44]. This will lead to an increasing application of WGS, which is of great value in outbreak analysis.

In conclusion, this study shows that although cgMLST provides a high discriminatory power in the epidemiological analysis of VREfm, transposon analysis increases the power of WGS and allows the detection of horizontal gene transfer. Combining these two methods allows to investigate both clonal spread as well as concomitant spread of MGEs which will lead to a better insight and understanding of the highly complex transmission routes during in-hospital and regional VREfm outbreaks.

Funding

This work was a part of the routine work.

Transparency declarations

None to declare

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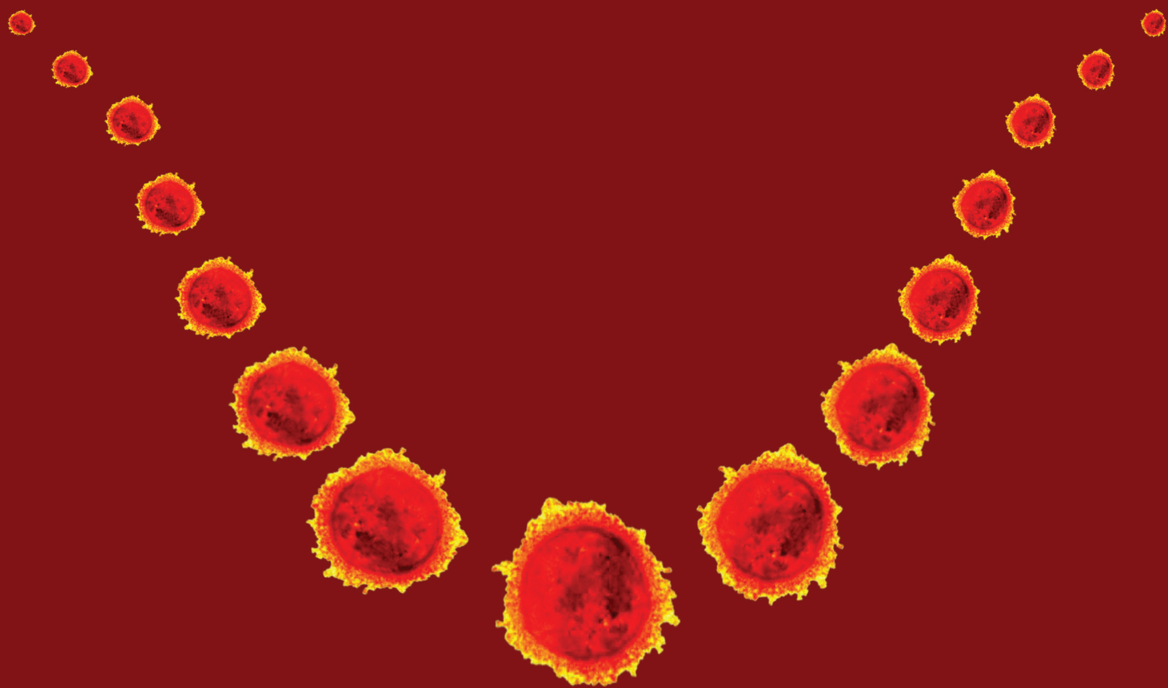

Summary, conclusion & discussion and future perspectives

8

SUMMARY

Enterococci already seemed to emerge as a leading cause of hospital-associated infections around 1970-80 [1]. Especially *E. faecium* rapidly evolved as a successful nosocomial pathogen [2], thereby causing infections in seriously ill patients, such as haemato-oncology patients [3, 4]. Moreover, the emergence of vancomycin-resistant-enterococci (VRE) is mainly due to successfully hospital associated (HA) *E. faecium* lineages (clade A1) that have acquired the *vanA* and/or *vanB* gene [5].

In this thesis we aimed to gain more insight in the evolution and epidemiology of *E. faecium* as described in Chapters 2, 3 and 6. These insights showed that several improvements are necessary for targeted (vancomycin resistant) *E. faecium* diagnostics, infection prevention, antimicrobial stewardship and typing methods. In Chapters 2 and 4-7 several of these specific innovations for (vancomycin resistant) *E. faecium* are studied and applied and have shown to be of value for patient care.

Chapter 1 contains a general introduction on this thesis. The origin of the enterococci are described as well as the rise of *E. faecium* as a nosocomial pathogen.

Chapter 2 continues to describe the background and evolution of *E. faecium*. *E. faecium* has acquired a collection of successful traits and easily adapted to several conditions, which has shaped this microorganism as the ultimate nosocomial pathogen of today. Based on these insights, implications and recommendations for infection control are given of which the most important are: 1) *E. faecium* is a highly tenacious microorganism by nature, which make them highly resistant to desiccation and starvation. This leads to prolonged survival in hospital environments. Enforced cleaning and disinfection procedures are needed combined with strict infection prevention measures to prevent further transmission. 2) Genetic capitalism of *E. faecium*: the continues refinement of genomic configuration, characterized by the flux and integration of successful adaptive traits, will result in a selective advantage and clonal expansion. This enormous genome plasticity makes that continuous awareness and epidemiological surveillance is needed to detect successful circulating strains and resistances to newer antibiotics and disinfectants.

In **Chapter 3** we studied the prevalence and molecular epidemiology of ESBL/plasmid mediated AmpC β-lactamase (pAmpC) Enterobacteriacae and HA *E. faecium* (including VRE) in hospitals in the Northern Dutch-German border region. In addition, stool community samples from the Northern Netherlands were screened for the same resistant pathogens. Dutch hospitals showed a prevalence for ESBL/pAmpC, VRE and ARE (ampicillin resistant/

HA *E. faecium*) of 6.1%, 1.3% and 23.6% respectively, whereas the prevalence in the community was 2.75%, 0.25% and 1.5%, respectively. The German hospital had an ESBL/pAmpC prevalence of 7.7% and 3.9% for VRE. Genetic relatedness by core genome multi-locus sequence typing (cgMLST) was found between two ESBL- *Escherichia coli* (*E.coli*) isolates from Dutch and German cross-border hospitals and between VRE isolates from different hospitals within the same region.

In **Chapter 4** of this thesis, we aimed to identify risk factors for the development of an *E. faecium* bloodstream infection (BSI) in patients with haematologic malignancies. Identified risk factors in this study were prior colonization with *E. faecium*, a combination of neutropenia and an abdominal focus, age >58 years, prolonged hospital stay (>14 days) and an elevated (C-reactive protein) CRP level (>125mg/L). Pre-emptive glycopeptide treatment can be applied to those haematology patients who are at high risk of developing an *E. faecium* BSI by using these risk factors in a risk stratification model. This allows antibiotic stewardship in terms of prudent use of glycopeptides which is helpful in controlling further spread of VRE.

In **Chapter 5** a PCR-based method, the Xpert *vanA/vanB* assay, was evaluated and optimized for the detection of *vanB* VRE carriage. To overcome false-positive results of *vanB* genes from gut anaerobes, the PCR was performed on overnight incubated enriched broth. This brain heart infusion (BHI) broth contained amoxicillin (16mg/L), amphotericin B (20mg/L), aztreonam (20mg/L) and colistin (20mg/L). The use of the Xpert *vanA/vanB* assay on these broths resulted in a decrease of C_T values for the majority of true-positive cases compared to the C_T value obtained from direct faecal samples. For true-negative cases, the opposite was observed as expected. Additionally, adjusted C_T cut-off values were used: a CT value of ≤25 for true positive cases and a C_T value of >30 for true negative cases. Samples with C_T values between 25 and 30 required confirmation by culture. This approach resulted in a sensitivity, specificity, positive prediction value (PPV) and negative prediction value (NPV) for detecting *vanB* VRE of 96.9%, 100%, 100% and 99.5%, respectively.

In **Chapter 6** various examples of diagnostic evasion mechanisms of highly-resistant microorganism (HRMOs) are given, each accompanied with practical laboratory detection advices. For VRE in particular, *vanB* VRE can easily remain undetected in routine diagnostics. In addition to the fact that fecal VRE carriage often is detected in very low amounts, vancomycin resistance in *vanB* VRE is not always expressed. VanB-type VRE isolates can have vancomycin MICs below the EUCAST susceptibility breakpoint of ≤4mg/L [6]. An important pitfall in VanA-type VRE is that isolates can be phenotypically susceptible

to vancomycin due to silenced *vanA* genes. These phenotypes of VRE can easily lead to uncontrolled outbreaks. We advise a combination of phenotypic (vancomycin disk diffusion, use of chromogenic agars) and molecular diagnostic (PCR) strategies in the detection of VRE.

The use of whole genome sequencing (WGS) to analyse VREfm outbreaks is described in **Chapter 7**. A total of 36 representative isolates of which sequence data were available from VREfm outbreaks that occurred in the University Medical Center Groningen (UMCG) in 2014 were typed by cgMLST by extracting the alleles from the WGS data. Additionally, *vanB*-carrying transposons of all sequenced isolates were characterised. CgMLST divided the 36 isolates into seven cluster types (CT); CT16 (n=1), CT24 (n=11), CT60 (n=1), CT103 (n=11), CT104 (n=8), CT105 (n=1) and CT106 (n=3). In addition, four different *vanB* transposon types were found. Within VREfm isolates belonging to CT103, two different *vanB* transposons were found, suggesting different outbreak events. On the contrary, VREfm isolates belonging to CT104 and CT106 harboured an identical *vanB* transposon, suggesting a single outbreak event. Clearly performing a combination of cgMLST and transposon analyses allows to investigate both clonal spread as well as the spread of mobile genetic elements (MGEs) which will lead to a better insight and understanding of the complex transmission routes in VREfm outbreaks.

CONCLUSION AND DISCUSSION

This thesis describes the evolutionary success of *E. faecium*, the rise of *E. faecium* infections as well as the emergence of VREfm worldwide. Based on the epidemiology and evolutionary insights we have come with practical tools and advices on different levels to withstand the further spread of successful hospital lineages of *E. faecium*.

Evolution and epidemiology of *Enterococcus faecium*

Concluding from several epidemiological studies, *E. faecium* has rapidly evolved as a successful nosocomial pathogen in the last two decades. As described in **Chapter 2**, evolutionary studies show that the emergence of *E. faecium* in hospitals is specifically due to strains belonging to subclade A1. The genome of *E. faecium* seems to be so flexible that it can easily adapt in response to environmental changes [7]. Through the continuous acquisitions and refinements of successful adaptive traits, also known as genetic capitalism, *E. faecium* lineages belonging to the hospital clade A1 has become the ultimate nosocomial pathogen. First, it became clear

that HA infections due to *E. faecium* rapidly emerged worldwide, largely replacing *E. faecalis* infections. Second, VREfm colonization as well as infections emerged as well. Regarding the evolutionary history of *E. faecium*, we foresee that the evolution of *E. faecium* will not stop. This pathogen will remain a challenge in hospitals in years to come, asking for a multi-facet approach and (cross-border) collaboration to optimize diagnostics, infection prevention and treatment of VREfm infections.

In **Chapter 3** of this thesis a secondary aim of the study was addressed: comparing the prevalence of AREfm and VREfm in the community and in hospitalized patients. No HA VREfm was found in the community samples. In addition, the number of AREfm in the community was low and only six ARE (6/400; 1.5%) were found, three of them being insertion sequence (*IS*) 16 positive. *IS*16 is a specific marker for hospital clades of *E. faecium* [8, 9]. In contrast, 23.6% of hospitalized patients were colonized with AREfm (105/445), all positive for IS16. This AREfm colonization was associated with antibiotic use. Normally, community associated (CA) clade B *E. faecium* strains predominate and outcompete clade A strains in the antibiotic free GI tract of humans in the community [10]. Although our study was not designed to detect clade B *E. faecium* strains, it does supports previous findings that colonization of HA *E. faecium* strains mainly occurs in a hospital environment. Acquisition through the hospital environment [11, 12] and antibiotic-induced outgrowth are both important factors herein. Especially the use of cephalosporin seems to be associated with AREfm [13, 14]. However also CA *E. faecium* strains are intrinsically resistant to cephalosporins. This implicates that there are additional effects [15] besides the antimicrobial effect of cephalosporins on the microbiome. Indeed, it is shown that there is also an immune response of the GI tract due to cephalosporins which makes that particularly clade A1 *E. faecium* are able to colonize the GI tract preceding antibiotic use [16].

As a result of its genomic plasticity, VREfm already developed several phenotypes difficult to detect, as shown in **Chapter 6**. This allows VREfm to evade diagnostics in order to become even more successful. The exact proportion of these evading phenotypes compared to wild-type phenotypes is not exactly known. For example, reported proportions of low-level *vanB* VRE carriage can range from 24.5% to 55% [17, 18]. Proportions of vancomycin variable enterococci (VVE) defined as *vanA*-positive, vancomycin-susceptible isolates can range from 15% in clinical and screening isolates in an outbreak setting [19] to 47% reported in sterile site isolates [20]. The therapeutic consequences of these evading phenotypes during antibiotic therapy are not exactly clear and depend on the chosen empirical therapy, but failure of therapy seems very likely in some of these phenotypes [21, 22].

Tailor made *Entercoccus faecium* tools and advices

Antibiotic stewardship is a key factor in preventing antibiotic resistance. In order to prevent the further spread of VRE, one of the therapeutic tools is the stringent use of glycopeptides. In **Chapter 4** of this thesis we aimed to develop a prognostic model in order to determine which haematology patients are at high risk of an *E. faecium* bloodstream infection (BSI) and in which empirical glycopeptide therapy should be given. Previous *E. faecium* colonization, neutropenia and abdominal focus of infection were the most significant risk factors. Other risk factors were advanced age, prolonged hospitalization and elevated CRP-level. We are aware that our study was a single centre study and that some of the risk factors found may be specific for our centre. However, especially previous *E. faecium* colonization has found to be a significant risk factor in other molecular epidemiological studies [4, 23]. Importantly, in this study no patients were found with VREfm BSI, though this prognostic model could be used to predict VREfm BSI in our institute as well. In fact, another study developed a similar clinical model to predict which haematology patients would develop VRE BSIs guiding the empirical anti-VRE therapy [24]. Previous colonization, neutropenia and mucositis were also included in their prediction model as they are in ours. Direct identification of *E. faecium* in positive blood cultures has become possible [25] in routine diagnostics, also in our centre. This reduced the turnaround-time and had a major impact on antimicrobial stewardship [26]. However, our model is still of use in the critical period before positive blood cultures.

The ability to evade diagnostics may be considered as a success factor in the emergence of VREfm lineages. In **Chapters 2 and 6** known evading VRE phenotypes are described, together with laboratory tools to detect them. Antimicrobial resistance creates significant clinical challenges. For this it is important to combine state of the art phenotypic and molecular laboratory diagnostics. For the latter, rapid and accurate molecular diagnostics would be ideal. The Antibacterial Resistance Leadership Group (ARLG) invests in innovations in new diagnostics [27]. For example, rapid molecular diagnostic (RMD) platforms to detect genes conferring to resistance/susceptibility to Acinetobacter spp. has recently been evaluated [28]. Still, in general, studies are needed to assess how these new diagnostics should be implemented, how they perform and whether they are cost-effective. Detection of VRE can be a challenge since microbiological laboratories should be aware of resistance mechanisms that are not detected by routine diagnostics. Reporting of alarming evading HRMOs via healthcare networks could be of help, together with specific diagnostic recommendations. Second, laboratories should have the diagnostic tools available. Laboratories often have their own diagnostic arsenal with major differences between

laboratories. This does not necessarily have to lead to diagnostic evasion, but laboratories that do not have access to state of the art diagnostic tools are at risk. For example, low-income countries might not always have access to molecular diagnostic tools.

In **Chapter 5** we have described a diagnostic tool, specifically adjusted to detect *vanB* VRE, including those that can evade diagnostics because they express low vancomycin MICs. An important goal of VRE diagnostics is that it can produce rapid and reliable results for clinical decision making [29]. Direct PCR on faecal samples can often result in false-negative results for *vanB* due to the presence of *vanB* genes from anaerobic bacteria residing in the gut [30, 31]. In this study, we adjusted the manufactures' guidelines concerning the cut-off C_T-values for positivity of their PCR assay. We used a cut-off C_T-value of ≤ 25 for positivity by PCR on enriched broths. For broths with C_T-values between 25-30, we recommend to confirm this by culture. C_T-values of >30 appeared to be true-negative. Our study showed that this is a useful tool in outbreak situations, since clear infection prevention measures can be taken based on these results. As noted above, laboratories need to evaluate the performance of their diagnostic tools and adjust their algorithms if necessary. Indeed, also for our tool there are still some improvements that can be made. First, metronidazole could be added to the broth to also inhibit the amoxicillin-resistant anaerobic bacteria. Second, the Xpert *vanA/B* cartridges are quite expensive and could lead to enormous costs in case of an ongoing VRE outbreak. It would be worthwhile to explore the alternatives and, for example, to develop an in-house PCR.

In VRE outbreak situations, rapid and accurate typing is required to investigate the genetic relatedness between patients' isolates. In **Chapter 7**, cgMLST was used to type VREfm outbreak isolates by extracting the data from WGS. Additionally, detailed characterisation of *van*-carrying transposons (mainly *vanB*) was performed to determine possible horizontal gene transfer. CgMLST provided a high discriminatory power in the epidemiological analysis of VREfm. Furthermore, transposon analysis was shown to have an additional value in the outbreak investigation and to be essential in cases where outbreaks are caused by the movement of particular MGEs. Since the acquisition of van genes can occur by different pathways, e.g by *de novo* acquisition from anaerobic gut microbiota [32] or through the exchange of large chromosomal fragments between VREfm and VSEfm [33], combining cgMLST and transposon analyses in VRE outbreaks is essential. Hereby both clonal spread as well as concomitant spread of MGEs is assessed which will lead to a better insight and understanding of the highly complex transmission routes during VREfm outbreaks. We are aware of the costs of WGS and the fact that not every laboratory has the ability to

implement it. Therefore, regional collaboration is crucial. Not only to share knowledge to combat resistance, but also to share experience on typing methods. In the end, this will be of benefit to all collaborating partners in case of an outbreak situation. Eventually, due to the increased use of WGS worldwide and the improving sequencing technologies and analysis tools, the cost will decrease [34]. Furthermore, the use of WGS in outbreaks can lead to more targeted infection control measures and thereby become cost-effective [35].

FUTURE PERSPECTIVES

E. faecium has been shown to possess a genome which is so flexible that it can easily adapt to environmental conditions and changes. *E. faecium* has become a hospital adapted pathogen in which evolution will never stop. This continuously evolution is seen on a large scale but also within the host. In hospitals in Australia and New Zealand a new endemic VREfm clone – sequence type 796- rapidly disseminated. Since the population structure of ST796 VREfm remained very clonal, the authors suggest that this clone has a survival advantage in hospitals over its predecessors [36, 37]. Indeed, these clones seem to be more tolerant to hand-rub alcohols [38]. Not only new endemic clones further adapting to its environment seem to emerge, also resistance to last-line enterococcal drugs is starting to rise. Resistance to linezolid [39, 40] , daptomycin [41, 42] , tigecyclin [43-45] and quinupristin-dalfopristin [46, 47] have been reported in *E. faecium*. Especially linezolid resistance seems to rapidly emerge in several countries [48-51]. Interestingly, *cfr* genes responsible for linezolid resistance are found in *Clostridium difficile* [51-53]. Like for *vanB* genes, *E. faecium* can acquire resistance genes from other species, of anaerobes in particular. Enterococci (predominantly *E. faecium*) and anaerobes may be left to dominate the microbiota after antibiotic treatment, for example cephalosporin's, and then exchange their genomic material. This underlines the importance to continue further epidemiological and evolutionary studies in *E. faecium*. These evolutionary studies may give us insights how to tackle this organism. For example, for the ST796 clone, specific attention should be paid to antiseptics. Additionally, both the role of clonal spread as well as the spread of MGEs should be investigated in *E. faecium* outbreaks. It would be of interest to investigate the presence of resistance genes in anaerobic bacteria and to determine which can be a potential donor for *E. faecium*.

Next to the efforts that need to be taken to control VREfm in which cross-border collaboration may play an important role, more research is needed to tackle the ongoing success of *E. faecium*. For example, the effect of administration of a (fecal) cocktail

containing microbiota clearing VREfm in humans colonised with VREfm would be interesting to investigate [54, 55]. This might reduce further transmission and dissemination of VREfm in hospitals. Also, innovations in the detection and typing of VREfm are needed. Examples are the development of better selective media, highly specific and clone-specific PCRs for each unique VRE outbreak event, and rapid point of care tests to detect VRE more efficiently.

Another topic to pay attention to is antibiotic tolerance, defined as bacteria that can persist during temporary lethal concentrations of antibiotics, without a change in their minimal inhibitory concentration (MIC) [56]. Biofilm infections and infections in immunocompromised hosts can create an opportunity for tolerance [56]. Antibiotic tolerance can affect multiple antibiotics and it facilitates the evolution to resistance [57]. Often secondary mutations involved in the bacterial stringent response are found [58]. Recently, it was found that *E. faecium* acquired mutations in the stringent response (*RelA* mutant) despite appropriate therapy within the bloodstream in an immunocompromised host [59]. As a consequence, this has led to antibiotic tolerance for linezolid, daptomycin and quinipristin-dalfopristin. Another example for within-host evolution, is the acquisition of (hetero)resistance to linezolid, daptomycin and vancomycin upon prolonged multidrug therapy, suggested to be caused by a novel *fabF* mutation encoding a fatty acid synthase [60]. These within-host studies have some important perspectives. First, laboratory diagnostics determining MICs may not be sufficient in antibiotic tolerant bacteria. Herefore, next to MIC testing the minimum duration of killing (MDK) may be used [56]. Since MDK testing is quite laborious for routine testing, the tolerance disk test (TD-test) which is a modified disk test, could be used [61]. The principle of the TD-test is that is promotes the growth of surviving bacteria in the inhibition zone once the antibiotic has diffused away. These are the tolerant and persistent bacteria. Second, once antibiotic tolerance or hetero-resistance has evolved, this could affect multiple other antibiotics, leaving no treatment options left. This asks for the clinicians awareness and the need to develop new antibiotics. Not only with antibiotics other targeting mechanisms [62] but also targeting biofilms. For example, an investigational compound (ADEP-4) successfully eradicated the biofilms of *relA* mutant *E. faecium* strains [59]. Last, observing the evolution of *E. faecium*, it is interesting to discuss whether the human environment (e.g. modern life, antibiotic use, hospital environment) has selected this successful pathogen or did it selected us human beings as the ultimate host in which it can continue his parasitic and ultimate evolutionary lifestyle.

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Nederlandse samenvatting

Dankwoord

Biografie/Biography

9

NEDERLANDSE SAMENVATTING

***Enterococcus faecium* en VRE**

Enterococcen zijn facultatief anaerobe gram-positieve bacteriën die in het maag-darm stelsel van de mens, andere zoogdieren, vogels, insecten en reptielen gevonden kunnen worden. Binnen de familie enterococcen zijn er zo'n 50 verschillende soorten, waarvan met name de *Enterococcus faecalis* en *Enterococcus faecium* klinisch relevant kunnen zijn bij de mens.

Een belangrijk gegeven is dat er binnen de *E. faecium* twee verschillende soorten subpopulaties zijn, namelijk de "onschuldigen" die als commensalen in de darm van (gezonde) mensen leven en de "opportunisten" die ziekteverwekkend kunnen zijn in ernstig zieke patiënten (met een verminderde afweer) en die we voornamelijk in de ziekenhuizen vinden. Dit proefschrift focust zich met name op deze opportunistische ziekenhuis gerelateerde *E. faecium*.

In de afgelopen twee tot drie decennia heeft de *E. faecium* zich tot een belangrijke en succesvolle ziekenhuis bacterie ontwikkeld. De bacterie heeft de capaciteit om zich uitermate goed en continu te kunnen aanpassen aan verschillende condities. Zo heeft de enterococ een dikke celwand, waardoor hij goed bestand is tegen uitdroging, voedingsarm milieu, hitte en desinfectie. Hierdoor kan hij uitstekend overleven in de ziekenhuisomgeving. Van nature is de *E. faecium* al resistent tegen verschillende klassen van antibiotica, zoals penicillines, cefalosporinen en (laag-level) aminoglycosiden.

Indien men een infectie door *E. faecium* wil behandelen, is de eerste keus een antibioticum van de klasse glycopeptiden. Dit kan vancomycine of teicoplanin (beide glycopeptide antibiotica) zijn. Echter, *E. faecium* kan ook resistentie verwerven tegen deze glycopeptide antibiotica en wordt dan vancomycine resistente *E. faecium* (VREfm) genoemd. De twee belangrijkste VRE soorten zijn Van-A en Van-B type VRE. Bij *vanA* VRE is er resistentie opgetreden tegen zowel vancomycine als teicoplanin, bij *vanB* VRE is er alleen resistentie tegen vancomycine. Sinds 2000 loopt de incidentie van VRE in Europa op, waarbij het grootste aandeel te danken is aan het succes van de *E. faecium*. In Nederland is de incidentie (nog) laag, maar zien we landelijk toenemende problematiek in de vorm van VRE uitbraken.

Dit proefschrift

Het doel van dit proefschrift is tweeledig. In de hoofstukken 2, 3 en 6 wordt meer inzicht verkregen in de evolutie en epidemiologie van *E. faecium*. De snelle opmars van *E. faecium* inclusief VREfm vraagt om specifieke interventies op het gebied van diagnostiek, infectiepreventie, antibiotica stewardship en typeringsmethoden. Op basis van de eerder verkregen inzichten worden er praktische handvatten gegeven die van toegevoegde waarde zijn voor de patiëntenzorg. In hoofdstukken 2 en 4-7 worden enkele van deze onderzochte en uitgevoerde interventies beschreven.

Hoofdstuk 2 geeft de achtergrond en de belangrijkste inzichten in de evolutie van *E. faecium* weer. *E. faecium* heeft een aantal succesvolle eigenschappen verworven en kan zich makkelijk aanpassen aan verschillende condities, waardoor het een ultieme ziekenhuis bacterie is geworden. Een aantal van de belangrijkste inzichten, implicaties en adviezen zijn: 1) *E. faecium* is van nature een erg hardnekkig micro-organisme, wat maakt dat ze resistent zijn tegen uitdroging en een voedingsarm milieu. Hierdoor kan de bacterie langer overleven in de ziekenhuisomgeving. Intensieve schoonmaak en desinfectie procedures in combinatie met strikte infectiepreventie maatregelen zijn daarom noodzakelijk om verdere verspreiding te voorkomen. 2) Het "genetisch kapitalisme" van *E. faecium* maakt dat hij zeer geraffineerd zijn genetisch materiaal kan afstemmen wat gekenmerkt wordt door de influx en integratie van succesvolle adaptieve eigenschappen. Dit maakt dat stammen die evolutionair gezien een voordeel hebben, zich kunnen uitselecteren en succesvol kunnen verspreiden. Epidemiologische monitoring (surveillance) is daarom noodzakelijk om deze succesvolle stammen, inclusief hun nieuw verworven eigenschappen zoals resistenties tegen antibiotica en desinfectiemiddelen, te detecteren. 3) Doordat *E. faecium* van nature al resistent is voor een aantal antibiotica klassen, kan de bacterie makkelijk uitgroeien onder antibiotica druk en resistentie verwerven tegen additionele antibiotica. Antibiotica stewardship en goede surveillance van VRE, kunnen helpen VRE controleerbaar te houden in de ziekenhuizen. 4) Er zijn VREfm stammen die detectie door de standaard laboratorium diagnostiek van VRE kunnen "omzeilen". Dit kan tot aanhoudende VRE uitbraken leiden, zonder dat dit opgemerkt wordt. Het is daarom enerzijds noodzakelijk verdachte patiënten frequent te screenen op VRE-dragerschap, anderzijds zal het laboratorium verschillende gecombineerde fenotypische en genotypische methoden moeten gebruiken om VRE te kunnen detecteren. 5) Ziekenhuis gerelateerde *E. faecium* stammen zijn geëvolueerd vanuit een gemeenschappelijk origine. Daarom verdient het de voorkeur om in het geval van uitbraken een typeringsmethode te gebruiken die een groot onderscheidend vermogen heeft,

zoals een methode die gebaseerd is op whole-genome-sequencing (WGS). Daarnaast heeft gedetailleerde analyse van het transposon, waarop de genen voor vancomycine resistentie kunnen zitten, een additionele waarde.

In **hoofdstuk 3** hebben we gekeken naar de prevalentie en moleculaire epidemiologie van extended-spectrum beta-lactamase (ESBL)/plasmide gemedieerde AmpC positieve bacteriën en ziekenhuis gerelateerde *E. faecium* (inclusief VRE) in de ziekenhuizen van de Noord-Nederlandse-Duitse grens regio. Daarnaast zijn feces samples van gezonde mensen van Noord-Nederland gescreend voor dezelfde resistente pathogenen. Nederlandse ziekenhuizen hadden een prevalentie voor ESBL/AmpC, VRE en ARE (ampicilline resistente/ ziekenhuis gerelateerde *E. faecium*) van 6.1%, 1.3% en 23.6% respectievelijk. In de Nederlandse gezonde populatie waren de prevalenties 2.75%, 0.25% en 1.5% respectievelijk. Het Duitse grens ziekenhuis had een ESBL/AmpC prevalentie van 7.7% en 3.9% voor VRE. Hieruit blijkt dat *E. faecium* duidelijk een ziekenhuis gerelateerde bacterie is, terwijl in het geval van ESBL-producerende bacteriën ook gezonde mensen drager kunnen zijn. Genetische verwantschap werd aangetoond tussen twee ESBL *Escherichia coli* (*E.coli*) isolaten van Nederlandse en Duitse ziekenhuizen en tussen VRE isolaten van verschillende ziekenhuizen binnen dezelfde regio.

Een interventie op het gebied van antibiotica stewardship werd onderzocht in **hoofdstuk 4**. Bij patiënten met een hematologische maligniteit werden risicofactoren voor het ontwikkelen van een *E. faecium* bloedbaan infectie geïdentificeerd. Patiënten met een verhoogd risico zouden in aanmerking kunnen komen voor pre-emptieve antibiotica therapie. De geïdentificeerde risicofactoren in deze studie waren: voorafgaande kolonisatie met *E. faecium*, een combinatie van neutropenie en een abdominaal focus voor het ziek zijn, leeftijd >58 jaar, langdurige ziekenhuis opname (langer dan 14 dagen) en een verhoogd C-reactive protein (CRP) waarde (hoger dan 125mg/L). Deze risicofactoren kunnen in een risico stratificatie model worden gebruikt om behandeling te starten bij patiënten met een hoog risico. Hierbij is antibiotica stewardship in de zin van het voorzichtig voorschrijven van glycopeptiden mogelijk, wat kan helpen om verdere verspreiding van VRE te voorkomen.

Een interventie op het gebied van diagnostiek werd onderzocht in **hoofdstuk 5**. Hierin werd een PCR (polymerase chain reaction) methode, de zogenaamde Xpert *vanA/ vanB* assay, geëvalueerd en geoptimaliseerd ten behoeve van de detectie van *vanB* VRE dragerschap. Eerder is gebleken dat directe detectie van *vanB* VRE in feces tot veel vals-positieve resultaten leidt door de aanwezigheid van *vanB* genen afkomstig van anaerobe bacteriën in de darm. Om deze "ruis' zoveel mogelijk te voorkomen, is er voor gekozen

om de PCR uit te voeren op overnacht geïncubeerde verrijkte bouillons, in plaats van op direct feces materiaal. Deze bouillon bevatte als basis “brain heart infusion” bouillon waarin de antimicrobiële middelen amoxicilline (16mg/L), amphotericine B (20mg/L), aztreonam (20mg/L) en colistine (20mg/L) waren toegevoegd. Het gebruik van de Xpert *vanA/vanB* assay op deze bouillons resulteerde in een daling van de C_T waarden voor het merendeel van de terecht-positieve gevallen vergeleken met de C_T waarden die verkregen werden van directe feces samples. Voor terecht-negatieve gevallen werd zoals verwacht het tegenovergestelde gezien. Daarnaast hebben we gebruik gemaakt van aangepaste C_T afkapwaardes: een C_T waarde van ≤25 werd aangehouden voor terecht-positieve gevallen en C_T waarde van >30 voor terecht-negatieve gevallen. Materialen met C_T waardes tussen de 25-30 dienden bevestigd te worden met kweek. Deze aanpak resulteerde in een sensitiviteit, specificiteit, positief voorspellende waarde en negatieve voorspellende waarde in de detectie van *vanB* VRE van 96.9%, 100%, 100% en 99.5%, respectievelijk.

In **hoofdstuk 6** worden voorbeelden gegeven van verschillende bijzonder resistente micro-organismen (BRMOs) die de laboratorium diagnostiek kunnen omzeilen. Elk voorbeeld wordt vervolgens voorzien van praktische adviezen op het gebied van laboratorium detectie. Voor VRE in het bijzonder, kan met name *vanB* VRE ondetecteerbaar blijven in de routine diagnostiek. Naast het feit dat detectie van VRE dragerschap lastig is door lage hoeveelheden hiervan in de feces, wordt de vancomycine resistentie in *vanB* VRE niet altijd tot expressie gebracht. VanB-type VRE isolaten kunnen een vancomycine minimale inhibitie concentratie (MIC) hebben die lager uitvalt dan het breekpunt van ≤4mg/L die door de EUCAST (European Committee on Antimicrobial Susceptibility Testing) is gesteld. Hierdoor lijkt het dus dat het om een gevoelige in plaats van resistente *E. faecium* stam gaat. Een belangrijke valkuil bij VanA-type VRE is dat isolaten fenotypisch gevoelig zijn voor vancomycine door “stil” gevallen *vanA* genen. Door kleine DNA fouten in dit gen, komt de resistentie voor vancomycine niet tot expressie. In enkele gevallen van vancomycine therapie, kunnen deze stil gevallen genen toch weer hun werk gaan doen. Deze omschreven fenotypes van VanA en VanB-type VRE kunnen beide leiden tot ongecontroleerde uitbraken. Daarom adviseren we om een combinatie van fenotypische (vancomycine disk diffusie, chromogene agars) en moleculaire diagnostiek (PCR) strategieën te gebruiken voor de detectie van VRE.

Een gecombineerde typeringsmethode voor VRE wordt beschreven in **hoofdstuk 7**. Zoals eerder beschreven is accurate en snelle typering in uitbraaksituaties noodzakelijk om genetische verwantschappen aan te tonen dan wel uit te sluiten. Dit om goede en snelle infectiepreventiemaatregelen te kunnen nemen. In deze studie wordt WGS toegepast om

VREfm uitbraken te analyseren. In totaal werden 36 VREfm isolaten waarvan de sequentie data beschikbaar waren van uitbraken in 2014 getypeerd middels core-genome multi-locus sequence typing (cgMLST). Daarnaast werden *vanB* bevattende transposons van alle isolaten gekarakteriseerd. Hiervoor werden data afkomstig van WGS gebruikt. CgMLST verdeelde de 36 isolaten in zeven cluster types (CT); CT16 (n=1), CT24 (n=11), CT60 (n=1), CT103 (n=11), CT104 (n=8), CT105 (n=1) en CT106 (n=3). Daarnaast werden er vier verschillende *vanB* transposons gevonden. Binnen de VREfm isolaten die tot CT103 behoorden, werden er twee verschillende transposons gevonden, wat suggereert dat het om twee verschillende uitbraken gaat. Aan de andere kant hadden de VREfm isolaten die tot CT104 en CT106 behoorden, een identiek *vanB* transposon, wat suggereert dat het om een enkele uitbraak gaat. Hierbij komt duidelijk naar voren dat de combinatie van cgMLST en transposon analyse beter inzicht geeft in de complexe transmissie routes bij VREfm uitbraken, omdat hierbij zowel klonale verspreiding als verspreiding door mobile genetische elementen (MGE) worden onderzocht.

Conclusie en toekomstperspectief

Samenvattend beschrijft dit proefschrift het evolutionaire succes van *E. faecium* en de opmars van VREfm. Op basis van epidemiologische en evolutionaire inzichten hebben we verscheidende praktische handvatten en adviezen gegeven om verdere verspreiding van de succesvolle ziekenhuis gerelateerde *E. faecium* stammen te weerstaan.

We voorzien echter dat de evolutie van deze bacterie nooit zal stoppen. Daarom is, naast meer samenwerking en surveillance, ook het optimaliseren van diagnostiek en onderzoek naar nieuwe behandelingsmogelijkheden nodig om het continue succes van *E. faecium* te tackelen.

BEGRIPPENLIJST

AmpC beta-lactamase = dit is een enzym behorende tot bepaalde klasse (klasse C) beta-lactamase dat bepaalde soorten antibiotica (penicillines en cefalosporines) kan afbreken

(facultatief) Anaeroob = (zowel met als) zonder zuurstof

Antibiotica stewardship = prudent/beleidvol/verstandig om gaan met antibiotica gebruik

cgMLST (core-genome multi-locus sequence typing) = hierbij worden DNA volgordes van een groot aantal genen van verschillende bacteriën met elkaar vergeleken, met als doel om naar de genetische verwantschappen te kijken

Chromosoom = drager van het erfelijk materiaal (DNA) van een micro-organisme. Bij bacteriën ligt dit in de vorm van ringvorming DNA los in (het cytoplasma van) de cel

Commensaal = een organisme dat in of op een gastheer leeft zonder deze te schaden of ziek te maken. Deze commensalen behoren tot de natuurlijke flora van een gezond lichaam

CRP (C-reactive protein) = zogenaamd acutefase-eiwit, wat toeneemt in het lichaam bij een ontsteking.

CT (cluster type) = verwante genomen van bacteriën die samen “clusteren” op basis van cgMLST

C_T waarde (cycle treshold) = het aantal cycli tijdens een PCR dat nodig is voor een signaal, hoe meer DNA wordt omgezet, des te sterker het signaal

ESBL (extended-spectrum betalactamase) = Dit is een enzym dat bepaalde soorten antibiotica (penicillines en cefalosporines) kan afbreken

Escherichia coli = een bacterie die in de darmen voorkomt, ook wel “poep” bacterie genoemd

EUCAST = Europese commissie die breekpunten/gevoeligheden voor antibiotica vaststelt. Dit wordt voor verschillende antibiotica en bacteriën gedaan

Fenotypisch = op uiterlijke kenmerken gebaseerd

Genotypisch = op DNA gebaseerd

Gram-positief = gramkleuring is een methode om bacteriën te kleuren om ze onder een lichtmicroscoop zichtbaar te maken. Gram-positieve bacteriën hebben een dikke celwwand, die tijdens de gramkleurig de (paarse) kleurstof vasthoudt

Moleculaire typering = hierbij worden op gedetailleerd niveau de verwantschappen van bacteriën met elkaar vergeleken op basis van genetische kenmerken. Men kan bijvoorbeeld kijken naar (een groot aantal) volgordes van genen. Een voorbeeld van moleculaire typering die men kan doen, is cgMLST

Incuberen = laten groeien van bacteriën (kweken) in een thermostaat

Isolaat = Een isolaat is een cultuur van bacteriën die zuiver is, in betekenis dat alle bacteriën in die cultuur afstammen van één enkele bacteriële kolonie, één bacteriële kolonie is een klein rond vlekje op een voedingsbodem ontstaan uit één enkele bacterie.

Klonaal = bij bacteriën betekent klonale verspreiding, verspreiding vanuit een voorouder waarbij al het genetisch materiaal wordt overgeërfd.

Kolonisatie = ook wel dragerschap, de aanwezigheid van micro-organismen op een anatomische lokatie, zonder daar ziek van de worden.

MIC (minimale inhibitie concentratie) = De laagste concentratie van een antibioticum waarbij de groei van de bacterie wordt geremd.

MGE = mobile genetische elementen, voorbeelden hiervan zijn een plasmide en een transposon, die los van het chromosomale DNA ook tussen bacteriën overdraagbaar zijn.

Neutropenie = tekort aan witte bloedcellen. Witte bloedcellen heeft een mens nodig voor een goede afweer.

Pathogeen = micro-organismen die ziekte-makend kunnen zijn

PCR (polymerase chain reaction) = is een manier om uit zeer kleine hoeveelheden DNA (enkele basen) specifiek een of meer gedeeltes te multipliceren (amplificeren) tot er genoeg van is om het te analyseren.

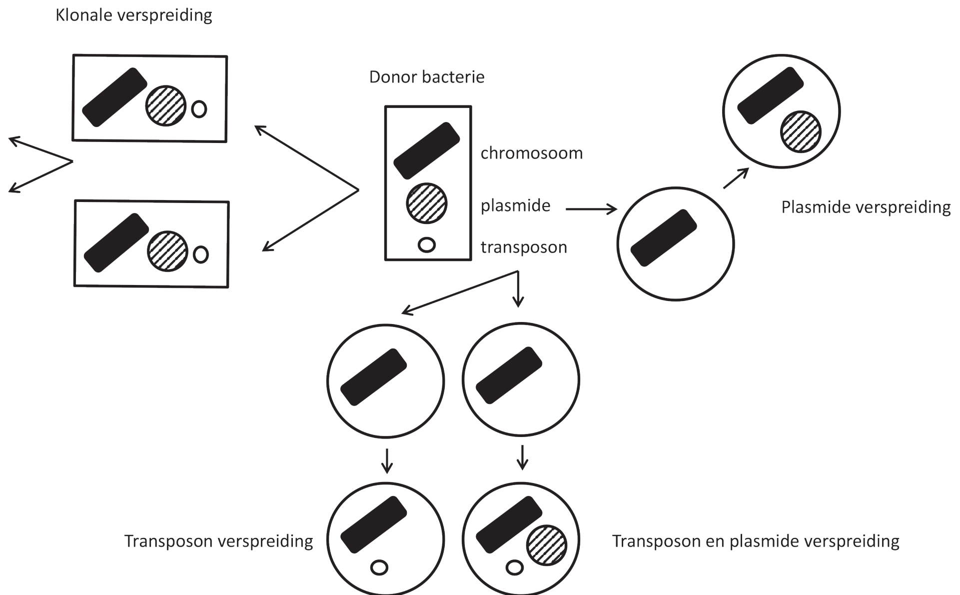
Plasmide = Een plasmide is een cirkelvormige streng DNA die zich buiten het chromosomaal DNA bevindt van sommige eencellige organismen. Met dit DNA kan genetische informatie tussen bacteriën, ook tussen soorten, worden uitgewisseld.

Pre-emptief = het preventief voordat de ziekte is vastgesteld, maar wel op basis van beleidsmatige criteria of risicofactoren, voorschrijven van antibiotica.

Transposon = ook wel springend gen (jumping gene) genoemd. Een stukje DNA wat in het genoom van plaats kan wisselen en wat, in geval van bacteriën, resistentie genen kan bevatten.

WGS (Whole-genome-sequencing) = sequencen is het bepalen van de volgorde van nucleotiden in de DNA-moleculen die het genoom vormen. In geval van WGS, wordt al het genetisch materiaal van een micro-organisme gesequenced.

Figuur 1: begrippen klonale verspreiding en verspreiding door mobile genetische elementen (MGEs) zoals plasmides en transposons



DANKWOORD

Na ruim 6 jaar is hier dan het eindresultaat in de vorm van een proefschrift. Wat eerst zo makkelijk leek door mijn enthousiasme, was zo makkelijk nog niet. Zeker de combinatie met een opleiding en gezin bleek toch iets pittiger dan gedacht. Zonder de hulp en steun van velen, inclusief collega's, familie en vrienden was dit nooit gelukt. Ik wil hen dan ook graag bedanken.

Allereerst veel dank aan Jan Arends. De wetenschappelijke stage bij jou was de start van een nieuw begin. Ik wil je bedanken voor deze fijne tijd en de inspirerende rol die je hebt gespeeld voor zowel het vak Medische Microbiologie als voor dit PhD traject, waar je een cruciale rol in hebt gespeeld.

Beste Alex, ik weet nog goed dat je bij mijn eerste presentatie kwam kijken waarin ik de resultaten van de wetenschappelijke stage presenteerde. Al snel daarna nam je mij aan als AIOS Medische Microbiologie en ben je promotor van mijn PhD project geworden. Je bent altijd enorm ondersteunend en motiverend geweest ("expose yourself"). Bezoeken aan congressen heb je altijd gefaciliteerd. Ik ben je ontzettend dankbaar voor alle mogelijkheden die je mij hebt gegeven, niet alleen tijdens mijn opleiding en PhD traject, maar ook voor de periode daarna, om te blijven als staflid.

John, ik ben ontzettend blij met jouw komst bij de afdeling en als promotor van mijn PhD project. Mijn proefschrift heeft daardoor een mooie wending gekregen wat in een aantal mooie manuscripten heeft geresulteerd. De begeleiding van de manuscripten was altijd heel prettig. Pragmatisch, kritisch en altijd met een glimlach. De laatste loodjes waren zwaar, bedankt dat je mij hier door heen hebt gesleept.

Mijn co-promotor Erik: je hebt ontzettend veel input geleverd en hield bovendien de grote lijnen van het manuscript goed in de gaten. Wat vond ik jouw snelle en behulpzame responsen fijn! Hierdoor kon ik, zeker in de laatste fase, vaart maken. Yes, we hebben de deadline die ik (uiteindelijk) gesteld had, gehaald! Veel dank hiervoor.

Heel erg bedankt voor de mooie analyses en fijne samenwerking, Monika. Lekker enthousiast als we waren en nog steeds zijn, hebben we een mooi werk geleverd en zijn we nog lang niet klaar. Laten we verder gaan samenwerken aan onze theorieën en nog meer mooi werk afleveren.

Ik weet nog dat mijn PhD traject begon met werken bij de Moleculaire bacteriologie (MolBac). Ik vind het nog steeds jammer dat het traject een andere wending heeft aangenomen Jan Maarten, maar desondanks wil ik je bedanken voor alle input, ideeën en steun. De tijd op het lab was ontzettend leerzaam en gezellig, mede dankzij alle (ex-)PhD studenten.

Bart Span, ik was aangenaam verrast toen ik hoorde dat ons algoritme nu ook in de praktijk wordt gebruikt. Bedankt voor jouw kritische blik en input.

I have learned a lot at the Robert Koch Institute Wernigerode. It was a good start for this PhD project and I had a very good time and enjoyed the city during Männertag. Thank you Guido and Carola for your time and experience. Wolfgang and Ingo, it was very nice to meet you.

Wat was het een klus om de prevalentiestudie op te zetten. Uiteindelijk hebben we er een mooie combinatie van gemaakt Silvia, Gijs, Dirk, Lieke, Nicole, Theo, Lesla, Robin Köck, Jan Weel en Jan van Zeijl. Thanks! Ook de andere betrokkenen, Wietske, Willy, Patrick, Marion, Norma, Aldert, Saskia, Age-Jan en alle andere verpleegkundigen en analisten die mee hebben geholpen met het includeren en verwerken van de patiënten en samples.

Hamideh, Brigitte en Pascal, bedankt voor jullie inzet om de Xpert *vanA/B* assay te evalueren. We zijn hier een stuk wijzer uit geworden en deze kennis wordt nog steeds gebruikt. Ook alle andere analisten die hierbij betrokken waren, dank.

Ook dank aan jou Sigrid, voor de analyses en de mooie opmaak van de figuren. Ieneke wat fijn dat je naar mijn manuscripten hebt willen kijken. Mariëtte, bedankt voor je input en speurwerk. Natasha, your data were so nice, let's continue that!

Rob Willems, het was een waar genoegen om samen met jou een review te schrijven waar je ontzettend goede en sterke elementen in hebt gezet.

De combinatie van opleiding en PhD was pittig en dat had je altijd goed door Greetje. Als opleider had je oog voor de balans tussen werk en privé. Heel erg bedankt voor je steun tijdens de opleiding, je vertrouwen, en de ruimte en ideeën die je creëerde om het allemaal net iets makkelijker te maken. Oud-opleider Annelies en de opleidingsgroep van Izore, dank voor jullie steun tijdens mijn opleiding en PhD.

Al mijn (ex-)collega's, Júlia, Lieke, Edwina, Claudy, Loredana, Carolien, Théke, Joppe, Lisa, Geesje, Anna, Wouter, Christien, Gro, Adrian, Nataliya, Marleen, Yanka, Marjolein, Nico, Bert, Bhanu, Kasper, Rik, Marjan en Coretta, bedankt voor jullie steun, gezellige kletspraatjes en etentjes. Hetzelfde geldt natuurlijk voor mijn collega's bij de infectiepreventie.

Mijn lieve paranimfen Nicole en Esther, bedankt voor het organiseren voor deze promotiedag. Nicole, wat fijn dat je dit allemaal doet terwijl je bijna tegelijk promoveert, maar zo houden we elkaar toch op de hoogte van alle checklijsten die er bestaan. De gezellige avonden waren en

zijn nog steeds onmisbaar. Esther, zo fijn dat jij er met een ervaren oog naar kijkt. De sushi dates zijn fijn om even werk te vergeten en gewoon te genieten.
Lieve vrienden en familie, het is dan nu helemaal afgerond: “dat drukke gebeuren, wat promotie heet”. Ik kan me voorstellen dat het niet altijd helemaal duidelijk is geweest waar ik mee bezig ben geweest. Maar jullie hebben voor een goede balans tussen werk en ontspanning gezorgd. Een aantal mensen wil ik in het bijzonder noemen:

Welmoed, Douwinde en Anna, onze avonden waren altijd ontzettend gezellig en geslaagd. Iris, we hebben leuke en gevarieerde vakanties samen doorgebracht die ik nooit zal vergeten. Ondanks dat we elkaar nu helaas een stuk minder zien, is onze vriendschap voor mij nog steeds ontzettend waardevol. Yvonne, wat was ik blij met jouw vriendschap toen we beiden weer in Leeuwarden waren gestrand. Wat heb ik ontzettend kunnen lachen met jou. Frederique, wat leuk dat we samen zwanger waren en nu alweer. Dorien, altijd luisterend oor voor mijn drukke gedoe, fijn dat je altijd flexibel bent geweest in het afspreken.

Lieve Yanwei, André en Mykah, onze bezoeken heb ik altijd erg gezellig en ontspannen gevonden. Zeker tijdens drukke perioden zorgden jullie ervoor dat ik even niet aan mijn werk hoefde te denken. Lieve Xiaowei, Bram, Thijmen, Kai en Lin, wat fijn dat jullie in Groningen wonen. Ik ben ontzettend dankbaar dat ik altijd welkom ben met Ize en dat onze kinderen dan lekker kunnen spelen. Hinke en Tienus, zonder jullie uitzonderlijke steun en inzet voor onze hele familie was ik nooit zover gekomen. Jullie zullen altijd een bijzonder plekje hebben.

Lieve papa en mama, al heb ik dan niet jullie restaurant overgenomen en is het voor jullie lastig te begrijpen wat ik doe, uit jullie reacties merk ik dat jullie trots zijn en dat maakt dat ik jullie gelukkige dochter ben. Bedankt voor jullie onvoorwaardelijke liefde en steun en alle normen en waarden die jullie mij hebben meegegeven die me tot de persoon hebben gemaakt die ik nu ben.

Mijn mooie dochter Ize met je dansende krullen, wat ben jij een geschenk. Zo vrolijk en lief dat jij bent, maakt mij een trotse en gelukkige mama. Iedere dag met jou is een belevenis en we gaan nog een hoop avonturen tegemoet met je toekomstige broertje. Mama houdt zielsveel van jullie.

Mijn liefste Frank, mijn stabiele factor. Wat ben ik blij dat ik jou heb ontmoet. Jij bent alles wat ik nodig heb. Dank je voor alle liefde, steun en ruimte die je mij geeft. Ik ben zo gelukkig met jou en ons gezin. Ik hou ontzettend veel van je en wil nog heel veel jaren met je doorbrengen.

BIOGRAFIE

Xuewei Zhou is geboren te Leeuwarden in 1987. Zij ging naar de middelbare school Piter Jelles Aldlân te Leeuwarden en in 2005 behaalde zij haar VWO diploma. In 2005 begon zij aan de studie Geneeskunde aan de Rijksuniversiteit Groningen. Na de junior co-schappen in het UMCG, zijn de senior co-schappen in het Medisch Centrum Leeuwarden doorlopen. Eén van deze co-schappen was bij de Medische Microbiologie van Izore, waar de interesse voor het vak ontstond.

In het laatste jaar van Geneeskunde begon Xuewei eerst aan haar wetenschappelijke stage bij de Medische Microbiologie in het UCMG onder begeleiding van Jan Arends (toenmalig arts-microbioloog) en Bart Span (internist-hematoloog). Het doel van deze studie was om risicofactoren te identificeren bij hematologie patiënten, die een verhoogde kans geven op een *Enterococcus faecium* bacteriemie en waarbij pre-emptieve antibiotica therapie gerechtvaardigd is. Dit heeft tot een artikel, het vierde hoofdstuk van dit proefschrift geresulteerd. Na deze wetenschappelijke periode, volgde nog de semi-arts stage bij de Interne Geneeskunde in het Martiniziekenhuis en de Klinische Genetica in het UMCG.

Contacten bij de Medische Microbiologie van het UMCG bleven. In 2012 startte de opleiding tot arts-microbioloog in combinatie met dit PhD-traject. Inmiddels is de opleiding succesvol afgerond en is Xuewei per augustus 2018 werkzaam als arts-microbioloog in het UMCG.

Xuewei Zhou was born in Leeuwarden in 1987. She attended the Piter Jelles Aldlân High School in Leeuwarden where she graduated in 2005. In 2005 she started to study Medicine at the Rijks University of Groningen. After her first year internships at the UMCG, she went to Leeuwarden for her second year of internships. One of these internships was Medical Microbiology at Izore where the interest for the profession started.

In the last year of Medicine, Xuewei started the graduation year with a Scientific project at the Medical Microbiology in the UMCG guided by Jan Arends (former Clinical Microbiologist) and Bart Span (internist-haematologist). The aim of the study was to determine risk factors for an *Enteroccocus faecium* bacteriemia in haematology patients and in whom pre-emptive antibiotic therapy should be started. This resulted in a publication, the fourth chapter of this thesis. Hereafter, the last internships were Internal medicine at the Martini hospital Groningen and Genetics at the UMCG.

Xuewei was still in contact with the Clinical Microbiology at the UMCG. In 2012 she started her training as a Clinical Microbiologist and combined the residency with this PhD project. Meanwhile, Xuewei has successfully finished her trainee and from August 2018 she works as a Clinical Microbiologist at the UMCG.