Summary, conclusion & discussion and future perspectives

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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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